Acta Haematologica

International Journal of Haematology   Journal International d'Hématologie   Internationale Zeitschrift für Hämatologie

Gegründet 1948 von K. Rohr † Zürich, und G. Rosenow New York

Organe Officiel de la Société Européenne d'Hématologie



1963

BASEL (Schweiz)   S. KARGER   NEW YORK

Contents – Sommaire – Inhaltsverzeichnis

Vol. 30



Printed in Switzerland by Buchdruckerei Aargauer Tagblatt AG, Aarau
Clichés: Steiner & Co., Basel

Acta haemat. 30 1 7 (1963)

From Paediatric Department and Institute of Clinical Biochemistry University Hospital, Oslo

Familial Vitamin B_{12} Malabsorption

By Olga Imerslund and Pål Bjørnstad

One of us (7) has formerly described 10 patients suffering from chronic relapsing megaloblastic anaemia with onset in infancy and childhood Eight of the patients also had permanent proteinuria without other signs of impaired renal function. There was a familial occurrence of the syndrome chronic relapsing megaloblastic anaemia and permanent proteinuria. These patients seemed to represent a specific, selective vitamin B_{12} malabsorption disease which had not been recognized as such before. The disease (and the syndrome) was assumed to be congenital and hereditary

The purpose of the present work has been to re-examine the 10 patients in order to attempt clarifying the pathogenesis and for this object we have used radioactive Co-labelled vitamin B_{12}.

Methods

The absorption of radioactive B_{12} has been measured by the method of Schilling (9) The assays have been performed in the standard manner using Co^{58} labelled vitamin B_{12} made by Philips "Duphar" Each oral test-dose has contained 1 microgram vitamin B_{12} with specific activity of 1 $\mu c./\mu g.$ The radioactivity has been counted in urine collected in 24 hours.

The presence of intrinsic factor First an ordinary Schilling test has been performed. Then the test has been repeated after an intrinsic factor concentrate was added to the oral test-dose. Finally the intrinsic factor activity of the gastric juice has been tested in bioassays on proved cases of pernicious anaemia (PA) From each patient sample of 80 ml. neutralized *gastric juice was administered by stomach tube to PA patient* immediately after an oral test-dose Co^{58} B_{12} and the amount of B_{12} radioactivity appearing in the urine during the next 24 hours was determined.

The intrinsic factor concentrate (IFC) was prepared by Abbot Laboratories from desiccated hog stomach. The IFC had been proved effective in assays on known PA.

The gastric juice from our patients was collected after intramuscular injection of 0.5 mg. histamine and filtered through gauze. pH was immediately adjusted to 10 with N NaOH to inactivate pepsin and after 20 minutes titrated to pH 7 with N HCL The juice was kept frozen at —20 °C until just before use.

Antibiotics, aureomycin or achromycin 250 mg were given perorally 3 times daily for 5 days. The SCHILLING tests were performed after 4 days of treatment.

Prednisone, 10 mg was given perorally 3 times daily for 6 days.

The SCHILLING tests were performed after 5 days of treatment.

Other laboratory methods were those described by IMERSLUND (7)

Material

The material consists of 6 males and 4 females. In the present paper they have the same designation as before with Roman numbers. They belong to 6 different sibships and families designated as A, B, C, D E and F (table I) Only siblings are affected, no parents or other relatives. No consanguinity has been demonstrated between the parents. However 3 couples have their origin from relatively isolated valleys in Norway

The onset of symptoms was from 5–6 months to 4 years of age (table I)

Table I

Age when symptoms started and when anaemia and proteinuria were discovered.

Family	Patient No.		Age when symptoms started years	Age when anaemia was discovered years	Age when proteinuria was discovered years
A	I	♂	1 $/_{12}$	1 /	$2^{*}/_{12}$
	II	♂	$/_{12}$	$/_{12}$	$/_{12}$
B	III	♂*	4	4 $/_{12}$	
C	IV	♂	1 $/_{12}$	2	2
	V	♀	1 $/_{12}$	1 $/_{12}$	1 $/_{12}$
	VI	♀	1 $/_{12}$	1 $/_{12}$	1 $/_{12}$
D	VII	♀	1 $/_{12}$	1 $/_{12}$	1 $/_{12}$
	VIII	♂	1	1 $/_{12}$	1
E	IX	♀	1 $/_{12}$ ($^{10}/_{12}$?)	2	2
F	X	♂	1 $/_{12}$	1 /	

A sister died from megaloblastic anaemia at the age of $5^{4}/_{12}$ years.

Symptoms and signs were such as are seen in vitamin B_{12} deficiency Pallor weakness, irritability gastrointestinal symptoms, glossitis or atrophic tongue loss of appetite, tendency to constipation or slight diarrhea. Slight or doubtful neurological changes were observed in three patients and premature greying of the hair in one.

Haematological findings: The anaemia was macrocytic with PRICE JONES curves of asymmetric heterogeneous type similar to those seen in pernicious anaemia. Tendency to moderate leucopenia, granulocytopenia and occasional thrombopenia was observed. The serum iron was with few exceptions increased, the icterus index normal or slightly increased. The bone marrow showed typical megaloblastosis with giant myelocytes and "stabcells

Findings in the urine: Eight of the 10 patients (table I) had permanent proteinuria (from trace to 2*) with minimal microscopic findings. By the concentration tests specific gravity was 1027—1036. Urea and creatinine clearances were within normal range

Urinary tract anomalies. Five of the 10 patients had urinary tract anomalies of relatively homogenous appearance which was regarded as representing different stages of duplication of the urinary pelvis and ureter from slight t complete.

Gastric juice Free hydrochloric acid was present in all aspirated EWALD test meals except once in one patient and always in gastric juice aspirated after injection of

betamine. The quantities of microbiologically determined vitamin B_{12} binding factor in the gastric juice were normal.

Perorally administered small amounts of vitamin B_{12} + normal pooled human gastric juice did not produce any rise of haemoglobin and red blood cells (experiments carried out in 3 patients).

Vitamin B_{12} in plasma examined partly in remission and partly in relapse was decreased and varied between 15—448 $\mu\mu$g./ml.

Stools. Several examinations for ova and parasites were negative. Fat balance tests showed normal absorption of fat, varying between 91—99%.

Glucose tolerance curves were normal.

Gastrointestinal X-rays after Ba contrast were normal.

Response to treatment with vitamin B_{12}. Intramuscular injections of vitamin B_{12} induced typical haematological reaction and remission. Monthly injections of 50—100 micrograms of vitamin B_{12} have maintained remission and well-being until the present time. Without specific antipernicious treatment the anaemia has always relapsed.

Present Investigation

The age of the patients varied from 7—25 years (table II). They were well. Growth and development were normal. Thus 7 of them were taller than the average and 3 of them shorter the shortest being in the 25 percentile. The intelligence was normal judged by their achievements in school and later on. No definite abnormalities were detected at the physical examination except for premature greying of the hair in the oldest patient. There was no hypertension or enlargement of the heart. There were no neurological symptoms or signs.

The haemoglobin, red and white blood cell count and the differential count were within normal range.

Findings in the urine: Eight of the 10 patients had proteinuria as before (from trace to 1%) with minimal microscopic findings and no growth by bacteriological examination. At the electrophoretic examinations the urinary proteins consisted mainly of albumin.

Renal function. By concentration tests the specific gravity rose to 1029—1038. The serum creatinine was normal.

Gastric juice: The volume was normal when compared with material of healthy individuals from the same age groups. pH varied between 1—2.

Serum proteins were normal (electrophoresis).

Results

The results of the SCHILLING tests are seen in table II. The data represent the percentages of the oral test-dose Co^{60} B_{12} excreted in the urine in 24 hours. Without intrinsic factor material the patients excreted between 0 and 1.4 % of the test-dose given. When IFC was added to the test-dose there was no significant increase in the excretion. There was no increase in the excretion after sterilization of the gut with achromycin (given to 5 patients). There was no increase in the excretion after prednisone therapy (given to 5 patients).

Table II

The results of the SCHILLING tests in our patients.

Pat. No.	Age years	% excretion of oral test dose $Co^{58}B_{12}$ in urine in 24 hours			
		Without IFC	With hog IFC	After achromycin therapy	After prednisone therapy
I	25	1.2	1.0		
II	7	1.3	1.9	0.4	
III	19	0.6	1.0		
IV	19	0	0		
V	14	0	0	0.2	
VI	14	0.2	0.2		0
VII	19	0.1	0.3	0 4	0.1
VIII	14	0.2	0.2		
IX	12	1 4	0.4	0.5	
X	11	0	0.3	0.8	0.3

Table III

The results of the SCHILLING tests in pernicious anaemia patients.

PA	% excretion of oral test dose $Co^{58}B_{12}$ in urine in 24 hours		
	Without IFC	With hog IFC	With gastric juice from our patients
T H.	0.8	10.0	10.4 (No. I)
L. H.	0.2	14.7	12.0 (No. II)
S. M.	1.3	13.6	19.0 (No. III)
F N.	0.8	13.8	17 4 (No. IV)
T H.	0.8	10.0	12.0 (No. V)
F N.	0.8	13.8	15.0 (No. VI)
T H.	0.8	10.0	14.0 (No. VII)
R. S.	1.5	16.6	14.0 (No. VIII)
J M.	0.9	16.5	11.0 (No. IX)
R. T	0.8	13.0	11.8 (No. X)

Table III demonstrates the intrinsic factor activity of the patients "gastric juice when tested on different PA patients. Without administration of intrinsic factor the PA patients excreted 0.2—1.5 % of the test-dose given. When hog IFC was added the excretion rose to 10—16 6 %. When the PA patients were given the test-dose $Co^{58} B_{12}$ + the gastric juice from our patients the PA patients excreted between 10 4—19 0 % of the test-dose. Thus the intrinsic factor activity in the gastric juice was normal when tested on proved pernicious anaemia.

At all investigations the results were the same in the patients with proteinuria as in those without. Treatment with antibiotics had no effect on the occurrence of proteinuria.

Discussion

The vitamin B_{12} deficiency is demonstrated by the megaloblastic bone marrow the low level of plasma vitamin B_{12} and the therapeutic response to intramuscular injections of vitamin B_{12}. The results of the SCHILLING test demonstrate that the cause of the vitamin B_{12} deficiency is decreased absorption from the gastrointestinal tract.

With regard to the pathogenesis we have to consider the following known causes of impaired absorption of vitamin B_{12}

1 *Lack of intrinsic factor* can be excluded as the gastric intrinsic factor activity is normal in bioassays on proved PA, a finding which fits with the negative response in our patients after IFC was added to the oral test-dose Co^{60} B_{12}.

2. *Utilization of the vitamin by intestinal micro-organisms and parasites* can be excluded. There was no increase in the absorption of the radioactive labelled vitamin B_{12} after treatment with antibiotics. No ova or parasites have been found in the stools.

3 *Gross pathological changes in the gastrointestinal tract* including strictures, anastomoses, blind loops and diverticulosis can be excluded because of the normal radiological findings and because the resorption was not improved after treatment with antibiotics.

4. *Coeliac disease or idiopathic steatorrhea* can be excluded because of normal resorption of glucose and fat. In addition we have the clinical evidence against the presence of multiple absorption deficiencies, the normal growth and development when treated only with parenteral injections of vitamin B_{12}.

5 *Immunological mechanism* are still an obscure cause of vitamin B_{12} deficiency However the fact that the vitamin B_{12} absorption was not improved after prednisone therapy points against immunological mechanisms playing any role.

As known causes of impaired absorption of vitamin B_{12} seem to be excluded, it seems reasonable to assume that the patients are lacking some specific intestinal factor (or factors) necessary for the normal absorption of vitamin B_{12}. Thus the so-called releasing factor demonstrated in the intestinal wall of the rat (3 6) may also be necessary in man. The lack of such a factor which probably is of enzymatic nature could be the cause of the vitamin B_{12} malabsorption in our patients.

Clark and Booth (1) and Dallman and Diamond (4) demonstrated lack of vitamin B_{12} absorption and vitamin B_{12} deficiency in 2 children aged 13 months and 3 years respectively after extensive resection of the distal small intestine in the newborn period. Apparently the clinical symptoms suggesting vitamin B_{12} deficiency in these patients had started at about 10 and 18 months of age. These observations support the previous assumption about the malabsorption being congenital in our patients, as the symptoms of vitamin B_{12} deficiency in most of them started in the same age group as in the afore-mentioned patients. The onset of symptoms probably depends upon the time required to empty the congenital stores of vitamin B_{12}. The situation seems analogous to the well known delayed development of vitamin B_{12} deficiency in patients after total gastrectomy. The fact that the results of the present investigation have been the same in the patients with proteinuria as in those without may fit in with the previous hypothesis that the proteinuria is an associated genetic phenomenon without primary pathogenetic importance for the vitamin B_{12} malabsorption.

It seems evident that our patients represent a new specific group of vitamin B_{12} malabsorption disease. To this group may belong the cases described by Gräsbeck et al. (5) as well as those described by Collé et al. (2) and by Lamy et al. (8). To the same group may also belong many of the incompletely examined cases formerly described as pernicious anaemia in infants and children.

Summary

A report is given on the results of the Schilling test in 10 previously described patients suffering from an apparently selective and specific vitamin B_{12} malabsorption disease. Without intrinsic factor material the urinary excretion of Co^{58} B_{12} was between 0 and 1.4% *of the test-dose administered. There was no significant increase in the* excretion when intrinsic factor material was added to the test-dose Co^{58} B_{12} neither after treatment with antibiotics (achromycin given to 5 patients) nor after prednisone (given to 3 patients). The intrinsic factor activity of the patients' gastric juice was normal when tested on patients with pernicious anaemia.

Résumé

Rapport sur les résultats obtenus à l'aide du test de Schilling chez 10 malades décrits auparavant déjà, souffrants d'une altération apparemment sélective et spécifique de l'absorption de la vitamine B_{12}. L'excrétion urinaire de la vitamine B_{12}-Co^{58} était de 0–1.4% de la dose-épreuve appliquée sans le facteur intrinsèque. Aucune augmentation significative de l'excrétion ne pouvait être observée après avoir ajouté le facteur intrin-

sèque à la dose-épreuve de vitamine B_{12}-Co^{58} ni après traitement par les antibiotiques (achromycine chez 5 malades) ni après application de prednisone (chez 3 malades). L'activité du facteur intrinsèque du suc gastrique des malades était normale (dosage chez des malades atteints d'une anémie pernicieuse).

Zusammenfassung

Es wird über die Ergebnisse mit dem SCHILLING-Test bei 10 früher beschriebenen Fällen von scheinbar selektiver und spezifischer Vitamin B_{12}-Resorptionsstörung berichtet. Ohne Intrinsic Faktor betrug die Urinausscheidung von Co^{58}-B_{12} 0–1.4% der zugeführten Testdosis. Es fand sich kein signifikanter Anstieg der Ausscheidung, wenn Intrinsic-Faktor der Testdosis von Co^{58}-B_{12} zugefügt wurde, weder nach Behandlung mit Antibiotika (Achromycin bei 5 Patienten) noch nach Verabreichung von Prednison (bei 3 Patienten). Der Magensaft der Patienten zeigt normale Intrinsic Faktor Aktivität bei Prüfung an Kranken mit perniziöser Anämie.

References

1. CLARK, A. C. L. AND BOOTH, C. C. Deficiency of vitamin B_{12} after extensive resection of the distal small intestine in an infant. Arch. Dis. Childh. 35: 595 (1960).
2. COLLE, E.; GREENBERG, L. AND KRIVIT, W. Studies of a patient with selective deficiency in absorption of vitamin B_{12}. Blood 18: 48 (1961).
3. COOPER, B. A. AND CASTLE, W. B. Sequential mechanisms in the enhanced absorption of vitamin B_{12} by intrinsic factor in the rat. J. clin. Invest. 39: 199 (1960).
4. DALLMAN, P. R. AND DIAMOND, L. K. Vitamin B_{12} deficiency associated with disease of the small intestine. Observations on an infant following extensive small bowel resection. J. Pediat. 57: 689 (1960).
5. GRÄSBECK, R.; GORDIN, R.; KANTERO, I. AND KUHLBÄCK, B. Selective vitamin B_{12} malabsorption and proteinuria in young people. Acta med. scand. 167: 289 (1960).
6. HERBERT, V.; COOPER, B. A. AND CASTLE, W. B. The site of vitamin B_{12}-intrinsic factor complex "releasing factor" activity in the rat small intestine. Proc. Soc. exp. Biol., N. Y. 110: 315 (1962).
7. IMERSLUND, O. Idiopathic Chronic Megaloblastic Anaemia in Children (Oslo Univ. Press, Oslo 1959). Acta paediat., Suppl. 119 (1960).
8. LAMY, M.; BESANÇON, F.; LOVERDO, A. AND AFIFI, F. La malabsorption spécifique de la vitamine B_{12} avec protéinurie. Arch. franç. Pediat. 18: 1109 (1961).
9. SCHILLING, R. F. Intrinsic factor studies. II. The effect of gastric juice on the urinary excretion of radioactivity after the oral administration of radioactive vitamin B_{12}. J. Lab. clin. Med. 42: 860 (1953).

Authors' address: Drs. Olga Imerslund and P. Bjørnstad, Rikshospitalet, Oslo (Norway).

Acta haemat. 30: 8-15 (1963).

Aus dem Czerny-Krankenhaus für Strahlenbehandlung der Universität Heidelberg
(Direktor: Prof. Dr. J. Becker)

Strahleninduzierte Blutveränderungen im Fluoreszenzbild

Von B. Chové

Die Verwendung von Vitalfarbstoffen zur Strukturanalyse biochemischer Zellveränderungen wird von dem Bestreben geleitet, intrazelluläre Reaktionen und Umsatzvorgänge möglichst unmittelbar optisch zu kennzeichnen. Die ersten Impulse zur vitalen Beobachtung von Zellstrukturen gingen von botanischer Seite aus (8, 9, 30, 31, 26). Besonders Strugger (26) hat die färbetechnische Bedeutung der Fluoreszenzindikatoren für die Protoplasmaforschung frühzeitig erkannt und zugleich auf die Brauchbarkeit der Methode zur Lösung strahlenbiologischer Probleme hingewiesen. Er benutzte bei seinen grundlegenden Färbeversuchen neben Pyronin und anderen Fluorochromen vorwiegend den Fluoreszenzindikator Acridin-Orange (AO), dessen Farbeffekt durch pH-Änderung variiert werden kann. Dieses Phänomen tritt durch den unterschiedlichen Konzentrationseffekt des Farbstoffes innerhalb der Zelle zutage, wobei im Normalzustand nur wenig, im Stadium der Zytolyse hingegen größere Mengen des in stärkerer Konzentration rot fluoreszierenden Stoffes aufgenommen werden. Diese Beobachtung gab den eigentlichen Anstoß zu ausgedehnten strahlenbiologischen Versuchen mit Alpha- und Röntgenstrahlen (28, 18, 3).

Die von botanischer Seite gewonnenen Ergebnisse bestätigten die Vermutung, daß bereits wenige Minuten nach Strahlenexposition eine Farbdissoziation infolge Membranschädigung der Zelle sichtbar wird. Allerdings waren hierfür Strahlendosen von über 100 000 r notwendig, deren Einwirkung in entsprechend kurzer Zeit erfolgen mußte.

Für die Anwendung der AO-Fluorochromierung auf medizinischem Gebiet waren ähnliche Gesichtspunkte maßgebend, ob-

gleich hier natürlich andere färbetechnische Voraussetzungen vorliegen. Einen relativ breiten Raum hat sich die Methode innerhalb der Zytodiagnostik erobert, wenngleich die Diskussion darüber noch nicht abgeschlossen ist (22 23 2, 6)

Die ersten fluoreszenzmikroskopischen Untersuchungen im Blut wurden von russischen Autoren aufgenommen (20) und in der Folge von gleicher Seite intensiviert (5 4 15 16) Bei den zunächst auf tierexperimenteller Basis durchgeführten Untersuchungen wurde die Vitalfärbung durch Zusatz von Kongorot (4) modifiziert, um die membrangeschädigten Zellen hervorzuheben. Die Ergebnisse differieren erheblich. Während Meisel und Sondak (20) bereits innerhalb einer halben Stunde nach Strahlenexposition «Zellnekrosezonen» im Knochenmark von Ratten und Mäusen nachweisen konnten fanden Brodskii und Suetina (5) keine nennenswerten Anzeichen für eine DNS-Depolymerisation. Die Diskrepanz der Befunde ist teilweise sicher technischen Ursprungs. Die AO-Färbung stellt infolge seiner starken pH Abhängigkeit und notwendigen Differenzierung zur Erzeugung der Metachromasie an den Untersucher hohe Anforderungen Ein schematisches Vorgehen ist nach unseren Erfahrungen auch bei Untersuchungen am gleichen Substrat nicht möglich denn schon geringe Änderungen der Präparatdicke fallen beim Färbeprozeß empfindlich ins Gewicht. Dadurch wird auch die vergleichende Betrachtung der Ergebnisse außerordentlich erschwert. Der gelungene Versuch einer Farbstandardisierung durch genaue pH Einstellung und Abstimmung des sogenannten Differenzierungsvorganges auf bestimmte Blutzellen (Granulocytenkerne) – Untersuchungen, die vor allem auf Schümmelfeder (23) und Bertalanffy (2) zurückgehen – hat die klinische Bedeutung der Methode erhöht.

In diesem Zusammenhang sollen nur die Aspekte der Supravitalfärbung im Hinblick auf strahleninduzierte Zellalterationen Berücksichtigung finden. Dabei interessiert speziell die Frage der Eignung dieser Methode als strahlendiagnostischer Frühtest.

Im Schrifttum werden die fluoreszenzmikroskopischen Möglichkeiten positiv beurteilt. Das Prinzip der Vitalfärbung erscheint auch aussichtsreich für die Erfassung zellulärer Frühschäden. Angeregt durch eine Publikation von Harding et al. (7) über postradiologische Blutveränderungen im Fluoreszenzbild in der – im Gegensatz zu Zellkernläsionen – über zytoplasmatische Farbdissoziationen (Verstärkung der Rotkomponente) berichtet wird, haben

wir analoge Untersuchungen an Strahlenpatienten durchgeführt. Gleichzeitig wurde ein größeres tierexperimentelles Programm mit Albinoratten gestartet. Die Ergebnisse sollen zusammenfassend diskutiert werden. Vorweggenommen sei die Feststellung daß die in die Fluoreszenzmikroskopie gesetzten Erwartungen nicht erfüllt wurden.

Methodik

10 Rattenkollektive von je 12 Tieren (Durchschnittsgewicht 200 g) eines hauseigenen Inzuchtstammes erhielten unter Tiefentherapiebedingungen (200 kV 1 mm Cu, FHA 1 m, Dosisleistung 25 *r/min in Körpermitte) eine einzeitige Ganzkörperbestrahlung* von 25 bis 200 Neben der einmaligen wurde eine iterativ-gestaffelte Strahlenexposition (ein- bis zweimal wöchentlich gleiche Strahlendosis) vorgenommen bei Ausdehnung des Beobachtungszeitraumes auf 4 bis 6 Wochen. In weiteren Bestrahlungskategorien wurden die Verhältnisse bis zur DL_{50} (300 bis 600 GKB) überprüft. Blutentnahmen erfolgten unmittelbar nach bis einschließlich 7 Tage nach Strahlenapplikation. Die Durchmusterung der Präparat wurde sowohl im Ausstrichpräparat als auch im Leukozytenkonzentrat (Blutentnahme aus der Vena femoralis) vorgenommen. Parallel fertigten wir Knochenmarksausstriche an. Die fluoreszenzmikroskopische Präparation erfolgte einheitlich nach der bereits früher angegebenen Technik (6) Im Differentialblutbild wurden neben den morphologischen Besonderheiten die Abweichungen im UV Bild unter selektiver Betrachtung der einzelnen Zellklassen (Lympho- Mono- und Granulocyten) registriert. Bei der Einstufung nach der zytoplasmatischen Aktivität der Zelle beschränkten wir uns auf insgesamt drei Stärkegrade, die mit 1 bis 3 klassifiziert wurden. Die Kategorie 0 umfaßt die Zellen ohne sichtbare Rotfärbung Um diese Farbunterscheidung überhaupt treffen zu können, ist es notwendig, eine optimale und stets gleichmäßige Farbdifferenzierung zu erzielen, die in der Praxis keineswegs garantiert ist Es gibt trotz Beachtung aller technischen Voraussetzungen Präparate die im UV Licht störende Reflexe oder Anfärbung des Untergrundes zeigen und damit für eine vergleichende Betrachtung ausscheiden. Abgesehen von diesem färbetechnischen Problem ist die zytologische Zelldifferenzierung im Fluoreszenzbild erschwert. Die Kernstruktur ist – wenigstens bei kleinen Kernvolumina – durch Überlichtungs unscharf. Ohne panoptisch gefärbten Parallelausstrich kann daher gar keine eindeutige Zelleinstufung stattfinden!

Bei entsprechender Berücksichtigung und Kenntnis dieser Besonderheiten ist eine exakte und vergleichbare Auswertungsgrundlage gegeben. Sie sollte ber nach möglichst objektiven Maßstäben (Blindversuch) vorgenommen werden.

Tabelle I

Zytoplasmatische Aktivität der Lymphocyten im Blut zu verschiedenen Zeitpunkten nach einmaliger *Ganzkörperbestrahlung* von *Ratten mit 50, 100 und 200 Auszählung von je 1000 Zellen in %*

Stärkegrad	0	1	2	3
Kontrolltiere (MW von 10)	28,3	62,6	7 46	1 46
Gruppe 1 3 Tage nach 50	23,03	64 75	10,35	1,83
Gruppe 1 7 Tage nach 50	25,25	69,95	4,2	0,6
Gruppe 2 3 Tage nach 100	18,55	62,55	17 1	1,8
Gruppe 2 7 Tage nach 100	28,0	63 45	7 45	1 1
Gruppe 3 3 Tage nach 200	42,63	36,5	15,33	5,5
Gruppe 3 7 Tage nach 200	21,2	69 15	7,55	2,1
Gruppe 4 14 Tage nach 200	29,6	62,5	6,5	1 1

Tabelle II

Fluoreszenzmikroskopische Differenzierung der Lympho- und Monocyten und Prozentwerte im Differentialblutbild bei fraktionierter Ganzkörperbestrahlung mit 25–50 bis zu einer Maximaldosis von 400 Vor Blutkontrolle 14 Tage bestrahlungsfreies Intervall.

Farbintensität	Lymphocyten				Monocyten			Differentialblutbild (%)			
	0	1	2	3	1	2	3	[illegible]	[illegible]	[illegible]	[illegible]
Gruppe 1 25 / Woche Gesamtdosis 100	11,2	64 1	17,3	7 4	7 7	46,2	46,1	50 1	33,0	16,5	0,4
Gruppe 2 2 × 25 / Woche Gesamtdosis 50	6,1	53,5	29 1	11,3	14,6	52,2	33,2	21,8	61,8	15,7	
Gruppe 3 50 / Woche Gesamtdosis 200	4,8	76,0	15 7	3,5	38,8	51,0	10,2	26,1	68,3	4,2	0,2
Gruppe 4 2 × 50 / Woche Gesamtdosis 100	11,7	59,5	22,9	5,9	15,5	57,6	26,9	38,8	50,5	9,7	0,4
Gruppe 5 25 / Woche Gesamtdosis 200	16,4	68,1	13,2	2,3	21,2	54,5	24,3	23,8	69,6	6,6	
Gruppe 6 50 / Woche Gesamtdosis 300	11,9	76,0	10,3	1,8	33,1	52,6	14,3	51 1	38,7	9,9	0,3
50 / Woche – 400	5,2	82,5	10,7	1,6	34,0	48,3	17 7	15,1	79,2	5,6	0,4
Gruppe 6a 50 r / Woche, Gesamtdosis 400 + 4 Wochen freies Intervall	7,3	68,5	17,6	6,8	13,7	60,2	26,1	32,1	60,2	7,3	0,3

Resultate

Unsere Resultate sind in den Tabellen I und II aufgeführt, je weils als Durchschnittswerte und getrennt nach den Zellformen im peripheren Blut. In Tab. I ist dabei auf die strahlensensiblen Lym phocyten Bezug genommen, bei denen 3 bis 14 Tage nach einmaliger Strahlenexposition mit 50 bis 200 r eine zytoplasmatische Dif ferenzierung der Farbstoffintensität durchgeführt wurde. Diese Zell gruppe zeigt normalerweise im überwiegenden Anteil einen schma len Rotsaum vom Stärkegrad 1 der offensichtlich in den Bestrah lungsgruppen 1 und 2 (50 bis 100 r) nicht faßbar verändert wird. Die erste Blutkontrolle nach 3 Tagen entspricht dabei dem morphologischen «Höhepunkt» der peripheren Blutveränderungen. Erst ab 200 r ist eine eindeutige Verschiebung der zytoplasmatischen

Aktivität zur Nullinie (42 65 gegenüber 28,3 /) feststellbar die 7 Tage nach dem Strahleninsult eine Art Gegenregulation aufweist und nach insgesamt 14 Tagen zur Norm zurückkehrt. In diesem Dosisbereich sind aber bereits charakteristische morphologische Kennzeichen einer Strahlenschädigung gegeben. In den unter schwelligen Bestrahlungsdosen von 25 bis 50 r finden wir auch bei Untersuchungen unmittelbar nach bis 24 h nach Strahlenapplika tion keine faßbare zytoplasmatische Aktivitätsschwankung gegen über der Norm. Dasselbe gilt für fraktioniert gegebene, unter schwellige Strahlenbelastung

In Tab II liegt eine kombinierte Auswertung der Lympho- und Monocyten bei mehrfacher Strahlenexposition mit 25 bis 50 r vor Zum Vergleich ist auch die quantitative Zellverteilung im Dif ferentialblutbild herangezogen Die ermittelten Werte lassen im Vergleich zur effektiven Strahlendosis keine signifikanten Abwei chungen erkennen. Hinzu kommt die beim Auftauchen strahlen induzierter Riesenzellen im Blut gegebene Verwechslungsmöglichkeit mit Monocyten oder ihre Fehleinstufung bei der Kategorie der gro-ßen Lymphocyten Die monocytären Blutelemente zeigen in der Regel eine lebhafte zytoplasmatische Aktivität, die eigentlich nur von den Plasmazellen übertroffen wird (was besonders bei Knochen-markausstrichen hervortritt) Eine fehlende Rotkomponente des Monocytenzytoplasmas konnten wir bis zur LD_{50} (600 r) nie fest stellen.

In Anbetracht dieser enttäuschenden Bilanz im peripheren Blut haben wir uns eingehenden Knochenmarkstudien zugewandt und unter gleichzeitiger Erhöhung der Ganzkörperdosis von 100 bis 700 r versucht, den zytoplasmatischen Früheffekt zu verstärken. Die vergleichenden und mikrophotographisch fixierten Farbaufnah-men fielen auch in dieser Richtung unbefriedigend aus. Allerdings wurde nur der Zeitraum bis zur akuten zellulär morphologischen Initialphase der Strahlenschädigung, also bis maximal 24 h post rad., als Wertmesser betrachtet.

Die Übertragung und der Einsatz der Methodik für klinische Fragestellungen, speziell zur diagnostischen Abklärung strahlen-therapeutischer Maßnahmen hat erwartungsgemäß keine Erwei terung des Blickfeldes gebracht. Eine Linksverschiebung des Blut bildes sowie atypische Blutzellen und Riesenzellformen treten zwar im Fluoreszenzbild farblich besser hervor sind aber für den auf

merksamen Betrachter auch im normalen Ausstrichpräparat nicht zu übersehen und zytologisch zweifellos sicherer einzuordnen.

Schlußfolgerungen

Die AO-Vitalfärbung als zytochemische Methode kann nicht als Empfindlichkeitstest zur Früherkennung einer Strahlenschädigung herangezogen werden. Die zytoplasmatischen Veränderungen (Schwankungen der Rotfluoreszenz) treten relativ spät ein und sind an eine Ganzkörperdosis von wenigstens 100 bis 200 r gebunden, können daher nicht als unmittelbare Strahleninduktionsfolge aufgefaßt werden. Die von Meisel und Sondak (20) beschriebenen Zellnekrosezonen oder ein Farbumschlag der Zellkern-Grünfluoreszenz in ein gelbliches Kolorit sind offenbar an unphysiologisch hohe Strahlenbelastungen gebunden, die für klinische Belange ohne Interesse sind. Trotz der einfachen Färbetechnik stößt das Fluoreszenzverfahren wegen der Notwendigkeit einer einheitlichen Farbabstimmung, die ja erst die Bezugsbasis für die Auswertung abgibt, auf erhebliche praktische Schwierigkeiten.

Über Strukturänderungen von Nukleinsäuren unter der Einwirkung einer ionisierenden Bestrahlung liegen einige experimentelle Arbeiten vor (5 21 29) Aus ihnen geht hervor daß der DNS-Komplex als Makromolekül außerordentlich strahlenresistent ist. Brodsky und Suetina (5) ermittelten für verschiedene kernhaltige Knochenmarkzellen einen annähernd gleichen DNS-Gehalt von 5,5 — 6,2 × 10^{-12} g während der RNS-Anteil mit zunehmender Ausreifung abnimmt Der Nukleinsäure /Nukleotid Index beträgt für die erythropoetische Reihe 2,5 bis 3 0 und für die myeloischen Zellen 1,5 bis 1 8. Eine im Tierversuch an Mäusen durchgeführte Letalbestrahlung mit 700 r hatte innerhalb von 24 h keine faßbare Reduktion des Nukleinsäurespiegels zur Folge. Hingegen zeigten dieselben, morphologisch noch intakten Zellen bereits unmittelbar nach dem Strahleninsult einen steilen Abfall der Nukleotidfraktion, die nach 6 h einen Nullwert erreichte und nach 24 h eine ansteigende Tendenz erkennen ließ Bei offensichtlich geschädigten Zellen (Kernfragmentationen und Nekrobioseformen) war gleichzeitig ein Absinken der Nukleinsäuren um den Faktor 3 bis 5 festzustellen.

Tirrlin et al. (29) postulieren zwei Reaktionsformen der DNS die auf Grund einer unterschiedlichen Molekülkonfiguration eine verschiedene Radiosensibilität aufweisen sollen. Aber auch aus diesen Untersuchungen kann nur der Schluß gezogen werden, daß

der strahleninduzierte Abbau der DNS-RNS-Moleküle zahlreiche metabolische Zwischenstufen durchläuft, die mittels der Acridin-Orange Fluorochromierung im Frühstadium nicht erfaßt werden können

Zusammenfassung

Anhand klinischer und tierexperimenteller Untersuchungen über den Wert der Acridin-Orange-Supravitalfärbung zur Frühdiagnose einer Strahlenschädigung werden Technik und Voraussetzungen dieser Methode einer kritischen Betrachtung unterzogen. Die negativen Ergebnisse rechtfertigen keine Anerkennung des Fluoreszenzverfahrens als strahlendiagnostischer Frühtest. Dieser Standpunkt trifft auf die Verhältnisse ein- bzw. mehrmaliger subletaler Strahlenapplikation zu und ist biochemisch zu begründen.

Summary

Technique and requirements for acridine orange supravital staining for the early diagnosis of radiation injury are critically examined on the basis of clinical and experimental investigations into the value of this method. The negative results do not justify the acceptance of the fluorescence procedure as a method of early diagnosis in radiation sickness. This conclusion applies to conditions of single and multiple sub-lethal exposure and can be explained biochemically

Résumé

A l'aide de données cliniques et de résultats obtenus par des expériences sur l'animal sur la valeur de la coloration supravitale par l'orange d'acridine pour le diagnostic supravitale par l'orange d'acridine pour le diagnostic précoce d'une lésion radiologique l'auteur soumet la technique et les conditions préalables de cette méthode à une étude critique Les résultats négatifs ne justifient pas l'adaptation d'une méthode de fluorescence comme test radiodiagnostique précoce. Ce point de vue vaut pour les conditions d'une applications unique ou répétée d'une dose sublétale de rayons-X et peut être justifié par la biochimie.

Literatur

1. Agroskin L. S. Present-day apparatus for cytospectrophotometry Biophysics 3: 323–333 (1958).
2. Von Bertalanffy L.; Masin M. and Masin, F A new and rapid method for diagnosis of vaginal and cervical cancer by fluorescence microscopy Cancer *11* 873–887 (1958)
3. Biebl, R. Weitere Untersuchungen über die Wirkung der Alphastrahlen auf die Pflanzenzelle. Protoplasma 35. 187 (1940).
4. Barvis, P V Fluorescence microscopy studies of the early changes induced in rat bone-marrow by chloroethylamines. Biophysics 3. 348–351 (1958)
5. Brodskii, V Ya. and Suetina, I A. Ultra-violet microscopy and cytophotometry of normal and X-irradiated bone-marrows. Biophysics 3. 83–93 (1958)
6. Choné, B. Dreidimensionale Tumorzelldiagnostik. Münch. med. Wschr *104* 621 623 (1962)
7. Harding, R. Stein A. and Mauro, J Post-irradiation changes in peripheral white blood cells observed with fluorescent microscopy Radiology *77* 282 285 (1961)
8. Hercik, F Die fluoreszenzmikroskopische Analyse der Alphastrahlenwirkung Protoplasma 32. 527 (1939).
9. Hercik, F Die Wirkung der Alpha Bestrahlung auf Zellgruppen. Radiologica 3. 214 (1938)

10. Hevesy G. Nucleic acid metabolism. Adv. biol. med. Phys. *1* 409 (1948)
11. Joshi, U. N. and Korgaonkar, K. S. Fluorescence of tissue culture cells stained with acridine orange. Nature *183* 400 (1959)
12. Kandor, L. B. and Litinskaya, L. L. Cytometric analysis of blood by electronic methods. Biophysics *5*. 820–830 (1960)
13. Khan-Magometova, I. D. Ultra-violet fluorescence by pyridoxin, indole and their derivatives. Biophysics *3*. 528–532 (1958)
14. Khan-Magometova, Sh. D. Gutkina, A. V. and Meisel, M. N. Ultraviolet fluorescence of certain animal organs and its change on irradiation. Biophysics *5*. 509–515 (1960).
15. Kroda, M. Ia. Method of fluorescence microscopy of peripheral blood. Biophysics *5*. 452 529 (1960).
16. Kroda, M. Luminescence microscopy of the peripheral blood following Roentgen irradiation. Med. Radiol. *4* 44–48 (1959)
17. Kraevskii, I. G. Significance of luminescence microscopy of the bone marrow in experimental studies of reactivity to radiation. Med. Radiol. *4* 49–52 (1959)
18. Krebs, A. Über die Verwendbarkeit der Struggerschen Akridinorange-Vitalfärbung für strahlenbiologische Probleme. Strahlentherapie 75. 346 (1944)
19. Krebs, A. T. and Gierlach, Z. S. Vital staining with the fluorochrome acridine orange and its application to radiobiology I. Alpha-ray effects. Amer J Roentgenol. 65. 93–96 (1951)
20. Meisel, N. M. and Sondak, V. A. Fluorescence microscope studies of early changes in bone marrow and blood of radiation-exposed animals. Biofiz. *1* 232 (1956)
21. Rapoport S. Ia. and Galanova, S. M. Mechanism of early radiation damage of bone marrow Biophysics *5*. 517 524 (1960)
22. Schümmelfeder, N. Zur histochemischen Bedeutung der Fluoreszenzmetachromasie des Acridinorange. Acta histochem., Suppl. *1* 140–151 (1958)
23. Schümmelfeder, N. Die Fluorochromierung des lebenden, überlebenden und toten Protoplasmas mit dem basischen Farbstoff Acridinorange und ihre Beziehung zur Stoffwechselaktivität der Zelle. Virchows Arch. *318*. 119–154 (1950)
24. Shapiro, I. M. The action of ionizing radiation on cell division an investigation of local and remote effects. Biophysics *3*. 446–453 (1958).
25. Strugger, S. Fluoreszenzmikroskopische Untersuchungen über die Aufnahme und Speicherung des Akridinorange durch lebende und tot Pflanzenzellen. Jena Z. Naturw 73. 97 (1940)
26. Strugger, S. In M. und H. Schaper's Fluoreszenzmikroskopie und Mikrobiologie (Hannover 1949)
27. Strugger, S. und Hilbrich, P. Die fluoreszenzmikroskopische Unterscheidung lebender und toter Bakterienzellen mit Hilfe der Akridinorangefärbung. Dtsch. tierärztl. Wschr. *50*. 121 (1942)
28. Strugger, S. P.; Krebs, A. T. and Gierlach, Z. S. Investigations into the first effects of Roentgen rays on living protoplasm as studied with modern fluorochromes. Amer J Roentgenol. *70* 365–375 (1953)
29. Tseitlin, P. I. Spitkovskii, D. M. and Riabchenko, N. P. Relation between molecular morphology of macromolecules of deoxyribonucleic acid and their radiosensitivity (problem of the radiosensitive and radioresistant forms of DNA) Biophysics *5* 454–459 (1960).
30. Zirkle, R. E. Relationships between chemical and biological effects of ionizing radiations. Radiology *52*. 846–854 (1949)
31. Zirkle, R. E. Some effects of alpha radiation upon plant cells. J. cell. comp. Physiol. *2*: 251–274 (1932).

Adresse des Autors: Dr. B. Chonè, Czerny-Krankenhaus für Strahlenbehandlung, *Heidelberg* (Deutschland).

Acta haemat. 30: 16–24 (1963).

From Medical Department C, Copenhagen County Hospital, Glostrup
(Chief Dr P From Hansen)

Histochemical Studies of Leukocytes from an Inflammatory Exudate

III. Di- and Triphosphopyridine Nucleotide linked Dehydrogenases

By Henrik R. Wulff

A variety of nucleotide linked dehydrogenases are now demonstrable by specific histochemical staining reactions, and in the present study such reactions have been used to investigate the pattern of oxidative metabolism in 'skin window' preparations of migrating leukocytes. A previous report concerned the activity of diaphorases and non-nucleotide-dependent dehydrogenases in similar preparations (15)

Methods

General procedure. The leukocytes were obtained by Rebuck's "skin window" technique (11). The cells migrated from small excoriation on the forearm or the thigh to cover slip placed on the lesion. The cover slip was replaced hourly for 12 13 hours. After removal from the lesion the preparations were dried for few minutes to avoid the non-specific dehydrogenase reaction (15) Subsequently they were incubated for 30 minutes at 37 °C, fixed in formol-saline and mounted in glycerine jelly Phase contrast examination was sometimes used for the identification of weakly or diffusely positive cells. Blood films were treated using the same procedure. The general technique has been described in detail previously (15).

Principle of histochemical reaction. The incubation medium contains the specific substrate of the enzyme, the appropriate coenzyme (diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) and tetrazolium salt. At the sites of dehydrogenase activity the coenzyme is reduced and formazan is deposited due to the subsequent diaphorase reaction (15)

Incubation media. The incubation media were essentially the ones recommended by Pearse (9) The standard medium was composed as follows substrate (0.1 M) DPN or TPN (0.01 M), sodium cyanide (0.01 or 0.001 M), magnesium chloride (0.005 M) Nitro Blue Tetrazolium (Nitro BT) (0.25 mg./ml.) polyvinyl pyrrolidone (75 mg/ml.) in phosphate buffer (0.015 M); pH 6.7—7 1 The standard medium was used to demonstrate DPN and TPN-linked *malic*, *lactic*, *isocitric* and *glutamic* dehydrogenase. I the remainder of the reactions the medium differed slightly DPN-linked *α-glycerophosphate*

Aided by a grant from "Arvid Nilsson Fond"

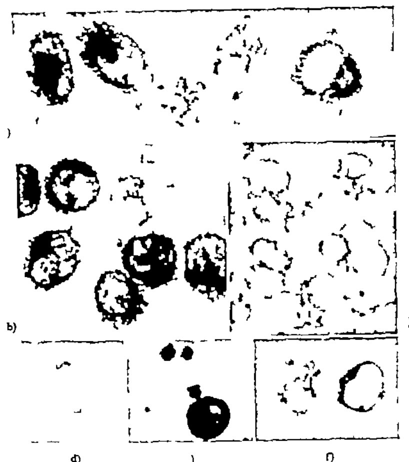


Fig. 1. a) DPN-linked malic dehydrogenase reaction in two neutrophils and three macrophages from "skin window" (10 hour preparation). b) DPN-linked lactic dehydrogenase reaction in macrophages and neutrophil from "skin window" (10 hour preparation). c) DPN-linked α-glycerophosphate dehydrogenase reaction in neutrophils from "skin window" (12 hour preparation). d) Weak reaction in macrophage from control preparation (DPN-containing standard medium). e) DPN-linked lactic dehydrogenase reaction in lymphocyte (and platelets) from blood film. f) DPN-linked malic dehydrogenase reaction in neutrophil and lymphocyte from blood film.

dehydrogenase: tris buffer (0.05 M) instead of phosphate buffer. *Glucose-6-phosphate dehydrogenase* (TPN-linked): tris buffer; sodium azide (0.01 M) and sodium fluoride (0.5 mM) instead of sodium cyanide. In some experiments 3(4,5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide (MTT) (0.25 mg./ml.) and cobaltous chloride (0.025 M) instead of Nitro BT. *β-hydroxy-butyric dehydrogenase* (DPN-linked): sodium azide instead of sodium cyanide. Control media were prepared omitting the specific substrate. In the DPN-linked α-glycerophosphate dehydrogenase series an additional control medium contained α-glycerophosphate, but DPN was omitted. The media

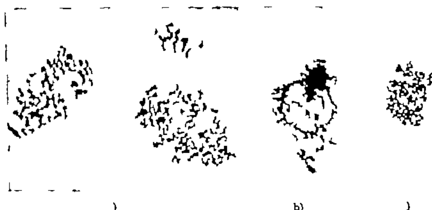
were stable for at least 12 hours, and were prepared from stock solutions of the different components (9), which part from the DPN and TPN solutions had been adjusted to pproximately pH 7 Owing to the acidity of the unadjusted DPN and TPN solutions it was necessary to adjust the final medium t a neutral pH using tris buffer (0.2 M) pH 9. Only DPN-linked malic and lactic dehydrogenase could also be demonstrated satisfactorily at a slightly acid pH. The control media were carefully adjusted to the same pH as the corresponding substrate-containing medium. I one experiment the TPN-linked lactic dehydrogenase medium was adjusted t pH 5.5 using 0.1 N HCl (5).

Material. Each enzyme was studied in preparations from 4—5 skin windows on 2—3 normal persons, except TPN-linked lactic dehydrogenase (pH 5.5) and TPN-linked malic dehydrogenase, each of which was studied in one skin window and TPN-linked lactic dehydrogenase (pH 7) and TPN-linked glutamic dehydrogenase, which were only studied in single preparations. I each experiment single preparations were incubated in the corresponding control medium.

Results

Both the pattern and the intensity of the formazan deposition varied when migrating leukocytes were stained for different nucleotide linked dehydrogenases. The strongest staining was obtained in the *DPN-linked lactic malic and isocitric dehydrogenase* series (fig 1a and b) The neutrophilic granulocytes which are the first cells to migrate, contained a moderate number of coarse, distinct formazan granules in the cytoplasm in addition to a weak diffuse background staining The nuclei were unstained The macrophages which appear 3 to 4 hours after the onset of the experiment, were more strongly positive Their cytoplasm contained numerous formazan granules which merged to form an almost diffuse purple precipitate The intensity of the reaction in both macrophages and neutrophils increased considerably with the progress of the inflammatory process to reach its maximum in the 8 to 10 hour preparations. The eosinophils contained fewer granules than the neutrophils, and basophils were not identified In the isocitric dehydrogenase series the macrophages were slightly less positive than in the lactic and malic dehydrogenase series and the deposits in the neutrophils were particularly coarse often forming short rods.

The inflammatory leukocytes stained less intensely for *DPN linked α-glycerophosphate and glutamic dehydrogenase* than for the preceding three enzymes. The first cells to migrate were often negative, but a positive reaction appeared after a few hours and in the last preparations both granulocytes and macrophages were moderately positive The neutrophils stained for α-glycerophosphate dehydrogenase only contained few granules, but the diffuse background staining was prominent (fig 1c)



) b))

Fig 2. Glucose-6-phosphate dehydrogenase reaction (Nitro BT) in skin window preparations.) Neutrophils from 12 hour preparation showing various degrees of activity. Two large, diffusely positive cells, one cell in which only part of the cytoplasm is positive (top right) and one cell, which is negative part from few isolated granules (bottom left). b) Macrophage from the same preparation.) Strongly positive eosinophil.

Control preparations from the later stages of the inflammatory process which were incubated in the DPN-containing standard medium without substrate presented a weak reaction especially in the macrophages, which, however did not reach the intensities obtained with the substrate-containing media (fig 1 d). The preparations from the α-glycerophosphate dehydrogenase series incubated in media without DPN or without substrate were negative. DPN linked *β-hydroxybutyric dehydrogenase* however was not demonstrated with certainty as the reaction did not differ significantly from that seen in the control preparations incubated in the azide-containing medium without substrate.

The preparations from the *TPN-linked malic and isocitric dehydrogenase* series were weakly stained in comparison with the ones stained for the corresponding DPN-linked enzymes. The first migrating cells were negative, and in the later preparations the macrophages only presented a weak cytoplasmic staining while the neutrophils contained few granules. No *TPN-linked lactic dehydrogenase* activity could be demonstrated at pH 5.5 and the staining at pH 7 was weak. The preparations examined for *TPN-linked glutamic dehydrogenase* were negative or almost negative.

In the *TPN-linked glucose-6-phosphate dehydrogenase* series both the pattern of activity and the intracellular localisation of formazan

differed from that seen in all the other series From the onset of migration some of the neutrophils were found to contain numerous closely packed formazan granules covering the whole cell, and it was usually impossible to distinguish the nuclear outline except by phase contrast examination (fig 2a) The positive neutrophils often belonged to the large type of cell most frequently encountered in early "skin window" preparations (14) The reaction in some of the eosinophils was even stronger than in the positive neutrophils (fig 2c) Many negative granulocytes were seen between the positive cells in contrast to all the other series where cells of the same type showed more or less the same intensity of staining The macrophages were weakly or moderately positive especially in later preparations, and the deposition of formazan in these cells did not differ appreciably from that seen in the other series (fig 2 b) When MTT was used instead of Nitro BT the same pattern of activity was seen but the reaction was weaker and the cobalt formazan granules were smaller and more distinct

The control preparations incubated in the TPN-containing media without substrate were negative Only in the controls from the glucose-6-phosphate dehydrogenase series some eosinophils presented a weak staining

The leukocytes in the blood films were damaged by the histochemical procedure and did not permit critical studies. In blood films stained for DPN linked lactic, malic and isocitric dehydrogenase the lymphocytes and monocytes were strongly positive and the neutrophils presented a weak staining (fig 1e and f) Some granulocytes stained diffusely for glucose-6-phosphate dehydrogenase, whereas the lymphocytes and monocytes were weakly positive Activity of DPN linked α-glycerophosphate, DPN linked glutamic and TPN linked isocitric dehydrogenase was only demonstrated in lymphocytes and monocytes.

Comment

The nucleotide linked dehydrogenases catalyse the oxidation of their specific substrate, while the coenzyme (DPN or TPN) undergoes reduction. The coenzyme may subsequently be reoxidised either by DPN or TPN diaphorase, which transfer the electrons to the cytochrome system, or by reductive dehydrogenase reactions requiring the same coenzyme. The histochemical reactions for

nucleotide-linked dehydrogenases imitate the former type of process both the dehydrogenase and the diaphorase participate in the reaction, and the tetrazolium salt acts as electron acceptor instead of the cytochromes. The deposits of formazan (reduced tetrazolium) therefore indicate sites of diaphorase activity associated with activity of the dehydrogenase in question. The overall activity of DPN and TPN diaphorase was reported in a previous paper (15)

The histochemically demonstrable enzymes from the tricarboxylic acid cycle are succinic, malic and isocitric dehydrogenase The activity pattern of DPN linked malic dehydrogenase resembled that of succinic dehydrogenase although the staining intensity was stronger (15) This supports the earlier interpretation that tricarboxylic acid cycle activity is greater in macrophages than in granulocytes and that it increases with the progress of the inflammatory process. The weak reaction obtained with the corresponding TPN containing medium is explained by a small affinity of malic dehydrogenase to TPN although malic decarboxylase, if present, may have contributed (8)

The strong DPN linked isocitric dehydrogenase reaction probably indicates that this enzyme is responsible for the greater part of isocitrate oxidation in leukocytes. Similar observations have been made in studies of other tissues (3) and DE DUVE AND HERS suggest that TPN linked isocitric dehydrogenase is not concerned with the tricarboxylic acid cycle but has other functions (2) In most histochemical studies, however the TPN linked enzyme has shown greater activity than the DPN linked enzyme (9)

Biochemical experiments have shown that suspensions of exudative neutrophils have a high lactate production and a low oxygen uptake (7) which suggests a high reductive lactic dehydrogenase activity and in comparison a low activity of diaphorases and tricarboxylic acid cycle enzymes. The present finding that the migrating neutrophils do not stain more strongly for lactic than for malic dehydrogenase may be due to the fact that DPN diaphorase participates in the histochemical demonstration of lactic dehydrogenase. It has, however been suggested by MARTIN et al. that the greater part of the lactate produced by neutrophils may not be formed by the reduction of pyruvate but by the action of glyoxylase (7) A TPN linked lactic dehydrogenase, which is more active at a weakly acid than at a neutral pH, has been demonstrated in phagocytising

leukocytes by KARNOVSKY (5) It was not demonstrable in skin windows using the present technique.

The glucose-6-phosphate dehydrogenase activity which was greatest in some of the granulocytes, may indicate glucose break down through the pentose shunt, but the enzyme may also be concerned with synthesis of ribonucleic acid or lipids. It is interesting that SMITH AND RUBINSTEIN found a high activity of this enzyme, TPN linked isocitric dehydrogenase and TPN diaphorase in tissue macrophages associated with necrosis, whereas activity of DPN linked dehydrogenases and DPN diaphorase was weak or absent (12) They interpreted their finding as a sign of lipid synthesis. The completely different pattern found in macrophages in "skin windows probably indicates that macrophage metabolism varies with the state of function it seems less likely that macrophages in different locations have different origins and different inborn enzyme patterns.

The function of DPN linked α-glycerophosphate dehydrogenase is uncertain It may participate in a glycerophosphate cycle with non nucleotide-linked (mitochondrial) α-glycerophosphate dehydrogenase, or it may be concerned with phospholipid synthesis (4 6) Glutamic dehydrogenase may also take part in various processes. The weak reaction observed in some of the control preparations, especially in the β-hydroxybutyric dehydrogenase series, was probably caused by endogenous substrate. Non specific reduction of the coenzyme ("nothing dehydrogenase activity) is less likely at the pH used in these experiments (9)

It is difficult to interpret the intracellular localisation of formazan in terms of subcellular morphology but it is apparent that the sites of enzyme activity in the case of glucose-6-phosphate dehydrogenase differed from that of all the other enzymes. The closely packed fine granular deposits observed in the neutrophils with this reaction seem to exclude a mitochondrial localisation. The result is in agreement with the observation from homogenisation studies that the enzyme is confined to the supernatant, but PEARSE states that it is mitochondrial in histochemical studies of tissue sections (9) The smaller variations in the size and shape of the formazan deposits in neutrophils observed in the other series possibly also reflect differences in the intracellular localisation of the enzymes Homogenisation studies have shown that DPN linked isocitric dehydrogenase is confined to the mitochondrial fraction, whereas DPN-linked α-

glycerophosphate dehydrogenase is found in the supernatant (8 13)

No satisfactory technique has yet been developed for the application of histochemical dehydrogenase methods to leukocytes from blood QUAGLINO AND HAYHOE recommend the use of acetone fixed blood films, but it is not known to which extent the various enzymes withstand this treatment (10) BALOGH AND COHEN altogether avoided fixation and were able to demonstrate a number of nucleotide-linked dehydrogenases in granulocytes, lymphocytes and monocytes (1) In the present study the greatest dehydrogenase activity was observed in lymphocytes and monocytes suggesting a higher oxidative metabolism in these cell types than in granulocytes.

Summary

The activity of nucleotide-linked dehydrogenases was studied histochemically in migrating granulocytes and macrophages from skin window preparations. The inflammatory cells stained intensely for DPN-linked lactic, malic and isocitric dehydrogenase. A weaker staining was obtained with reactions for various other dehydrogenases, including TPN-linked isocitric dehydrogenase. The intensity of most of the staining reactions increased with the progress of the inflammatory process, and usually the macrophages were more positive than the granulocytes. Only glucose-6-phosphate dehydrogenase presented pattern of activity different from that of all the other enzymes. For comparison the histochemical reactions were also applied on leukocytes from blood films. The results were discussed in terms of leukocyte metabolism.

Résumé

Détermination histochimique des déhydrogénases fixées aux nucléotides de granulocytes et macrophages émigrés des préparations des fenêtres cutanées. Les cellules inflammatoires montraient une coloration plus intense des déhydrogénases fixées aux nucléotides de l'acide lactique malonique et isocitrique. Les réactions avec diverses autres déhydrogénases, la déhydrogénase fixée au TPN de l'acide isocitrique incluse montraient une coloration plus faible. L intensité de la plupart des réactions de couleurs augmentait avec l'évolution du processus inflammatoire. Les macrophages réagissaient en général plus fortement que les granulocytes. Seule l'activité de la déhydrogénase du glycose-6-phosphate montrait une réaction différente de tous les autres ferments. A titre de comparaison, des réactions histochimiques sont pratiquées sur des leucocytes de frottis du sang périphérique. Discussion des résultats en relation avec le métabolisme leucocytaire.

Zusammenfassung

An ausgewanderten Granulozyten und Makrophagen aus «Hautfenster»-Präparaten wurde die Aktivität der Nukleotid-gebundenen Dehydrogenasen histochemisch untersucht. Die Entzündungszellen zeigten eine intensive Färbung für die DPN-gebundenen Dehydrogenasen von Milchsäure Äpfelsäure und Isocitronensäure. Eine schwächere Färbung fand sich mit Reaktionen auf verschiedene andere Dehydrogenasen, einschließlich der TPN-gebundenen Isocitronensäure-Dehydrogenase. Bei den meisten

Farbreaktionen nahm die Intensität mit dem Fortschreiten des Entzündungsprozesses zu, und im allgemeinen reagierten die Makrophagen stärker positiv als die Granulozyten. Nur die Glukose-6-phosphat Dehydrogenase zeigt ein von allen anderen Enzymen abweichendes Verhalten ihrer Aktivität. Zum Vergleich wurden die histochemischen Reaktionen auch an Leukozyten aus Blutausstrichen angestellt. Die Ergebnisse werden im Hinblick auf den Leukozyten-Stoffwechsel besprochen.

References

1. Balogh, K. and Cohen, R. B. Histochemical demonstration of diaphorases and dehydrogenases in normal human leukocytes and platelets. Blood *17* 491 (1961)
2. de Duve, C. and Hers, H. G. Carbohydrate metabolism. Ann. Rev. Biochem. *26*, 149 (1957)
3. Ernster, L. and Navazio, F. Studies on TPN-linked oxidations. I. Pathways of isocitrate oxidation in rat liver mitochondria. Biochim. biophys. Acta *26*, 408 (1957)
4. Estabrook, R. W. and Sacktor, B. α-Glycerophosphate oxidase of flight muscle mitochondria. J. biol. Chem. *233*, 1014 (1958)
5. Karnovsky, M. L. Metabolic shifts in leucocytes during the phagocytic event; in CIBA foundation study group No. 10. Biological activity of the leucocyte, p. 60 (Churchill, London 1961).
6. Kennedy, E. P. Metabolism of lipides. Ann. Rev. Biochem. *26*, 119 (1957).
7. Martin, S. P., McKinney, G. R. and Green, R. The metabolism of human polymorphonuclear leukocytes. Ann. N. Y. Acad. Sci. *59*: 996 (1955)
8. Nachlas, M. M., Walker, D. G. and Seligman, A. M. The histochemical localization of triphosphopyridine nucleotide diaphorase. J. biophys. biochem. Cytol. *4* 467 (1958)
9. Pearse, A. G. E. Histochemistry, Theoretical and Applied (Churchill, London 1960).
10. Quaglino, D. and Hayhoe, F. G. J. Acetone fixation for the cytochemical demonstration of dehydrogenases in blood and bone marrow cells. Nature *187* 85 (1960)
11. Riis, P. and Wulff, H. R. Dynamic studies of the relation between intravascular and inflammatory neutrophils in normal subjects. Acta haemat. *23*, 276 (1960)
12. Smith, B. and Rubinstein, L. J. Histochemical observations on oxidative enzyme activity in reactive microglia and somatic macrophages. J. Path. Bact. *83*, 572 (1962)
13. Tung, T. C., Anderson, L. and Lardy, H. A. Studies on particulate α-glycerophosphate dehydrogenase of muscle. Arch. Biochem. Biophys. *40* 194 (1952)
14. Wulff, H. R. Histochemical studies of leukocytes from an inflammatory exudate. Glycogen and phosphorylase. Acta haemat. *28* 86 (1962)
15. Wulff, H. R. Histochemical studies of leukocytes from an inflammatory exudate. II. Succinic dehydrogenase, mitochondrial α-glycerophosphate dehydrogenase and di- and triphosphopyridine nucleotide diaphorase. Acta haemat. *29*: 208 (1963)

Author's address: Dr. H. R. Wulff, Medical Dept. C, Copenhagen County Hospital, *Glostrup* (Denmark).

Acta haemat. 30, 25–34 (1963).

Department of Medicine University of Colorado School of Medicine and the Belle Bonfils Memorial Blood Bank, Denver Colorado

Inactivation of Pathological Inhibitors of Intrinsic Thromboplastin* by Procoagulant from Human Urine**

By Kurt N von Kaulla and Edith von Kaulla

The ineffectiveness of blood transfusions in patients with circulating inhibitors of intrinsic thromboplastin creates serious therapeutic problems during bleeding episodes. No drug neutralizing these inhibitors is known. In this report it will be shown that a constituent of normal human urine counteracts such pathological inhibitor activity of human plasma in vitro Some properties of this material have been described earlier (1 2) Its activity against circulating pathological inhibitors of intrinsic thromboplastin has been observed only recently

Materials and Methods

Procoagulant from human urine (abbreviated in the following as "T" fraction; see also footnote on page 00) was obtained with recently improved and simplified extraction procedure 2.5 liters of pooled urine from healthy males collected during the day flows by gravity (190 cm. drop) through Coors filtering thimble # 761/1 during he night. "T" fraction and urokinase are retained by the thimble and separately eluted from it by back-washing procedures outlined diagrammatically in fig. 1 The "T" fraction eluate is cleared by centrifugation (R.C.F of 1590 for 15 minutes) concentrated to the desired range by perevaporation in rarefied N atmosphere, dialysed against buffered saline pH 7 4 sealed in ampules, sterilized for 20 minutes in boiling water and deep frozen. A lyophilized powder can be obtained from the dialysed isoelectric precipitate forming in the eluate t pH 3.5 after acidification (with 0.1 N HCl).

Demonstration of circulating inhibitors of intrinsic thromboplastin. Abnormal thromboplastic activity brought about by circulating inhibitors was suspected in 5 out of 7 patients vailable for testing of the "T" fraction when poor prothrombin consumption, in presence of normal thrombin times (3), coincided with the inability to control bemor

* With this term no distinction is made as to whether the inhibitors interfere with intrinsic thromboplastin itself or with factors required for its formation.

** Read in part at the annual meeting of the Amer Soc. of Hematology Columbus, Nov 1962. Supported by grants-in-aid from the Belle Bonfils Memorial Blood Bank in Denver and U.S. Public Health Service (HE-05538)

rhage with repeated transfusions of fresh blood or fresh frozen plasma. The remaining two patients had minor bleeding episodes not requiring transfusion together with deficient prothrombin consumption. The abnormal inhibitor activity was confirmed when neither addition of at least 10% fresh normal plasma with good consumption, 10% BaSO adsorbed plasma, nor 10% aged serum corrected prothrombin consumption in the patients plasma. The plasma mixtures were standing at room temperature (20–25 C) for 5 minutes before recalcification. With high titer of inhibitor activity there was no correction after addition of 50 to 80% normal plasma. As a representative example for demonstration of a high inhibitor titer in the plasma tested with "T" fraction, the serum prothrombin times of patient R. C. after various additions are given in table I

Table I

Recalcification times and serum prothrombin times after various additions to patient R. C. plasma with high titer of circulating inhibitor of intrinsic thromboplastin.

Plasma	Additions	Recalc. time minutes	Serum prothr. time seconds
patient	none	93	16.8
patient	5% brain cephalin	87	15.6
patient	20% serum	36	16.8
patient	20% BaSO adsorbed plasma	57	19.6
patient	10% normal plasma	66	16.0
patient	20% normal plasma	77	17.3
patient	50% normal plasma	18	17 7
patient	80% normal plasma	11	24.2
normal contr	—	12	56.4

According to (4) diluted in buffered saline. 24 hours room temperature, then frozen. Fresh normal human oxalated plasma adsorbed on 50 mg $BaSO_4$/ml. same plasma, citrated. Note depressed prothrombin consumption in mixture containing 20% patient plasma and 80% normal plasma.

Prothrombin consumption test. Serum prothrombin time was measured in recalcified fresh citrated plasma as described earlier (5) In this particular method, the dilution of plasma during recalcification is kept at a minimum. This increases the sensitivity for inhibitor activity and produces normal recalcification times in the same range as the Lee White times.

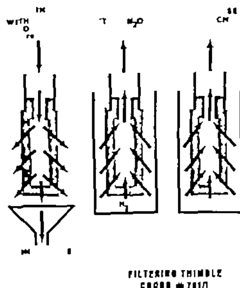
Thrombin generation test. This test was carried out in citrated plasma within thirty minutes after drawing of the citrated blood (3).

Thrombelastograms: Continuous recordings of the clot formation in recalcified citrated plasma (5) were obtained with the thrombelastograph (6)

Buffered saline pH 7 4 One part barbital acetate pH 7 4 (7) and four parts saline were mixed.

Addition of "T" fraction. For correction of clotting by "T" fraction in the tests above volume of the compound solution equivalent to 5 % of the plasma to be tested and corresponding to about 0.7–1 ml. urine per ml. plasma was added. The centrifuged "T" fraction filtrate (fig. 1 diagram II) or partially rediluted concentrated eluate, both after several hours dialysis in the cold against buffered saline were applied. For the thrombelastographic studies of fig 2, 1% solution of the lyophilized material was used increasing the volume of the plasma by 0.5 and 1 respectively All "T" fraction solutions were added within ten minutes before recalcification.

Topical application of "T" fraction. A 10 times concentrated sterilized thimble eluate occasionally prepared from the patient own urine was used. *Application* technique indicated in table II.



FILTERING THIMBLE
COORS # 761/1

Fig. 1. Separation of "T" fraction and urokinase from human urine. Diagram I represents retention of "T" fraction and urokinase by the thimble from the filtering urine. Diagram II indicates that only the "T" fraction is eluted by water which is sucked back through the thimble (first 20 ml. removing urine residues to be discarded, then 140 ml. containing the "T" fraction eluate). Diagram III shows the subsequent urokinase elution by 1 M KSCN (20 ml. to rinse thimble followed by 140 ml.).

Results

a) Effect of "T" fraction on hemophilic blood. The production of a firm clot within a normal time in hemophilia A plasma by 0.0015–0.003 mg/ml. "T" fraction is demonstrated with the thrombelastograms of fig. 2. The corrective effect of "T" fraction never failed with hemophilia A plasma.

b) Clinical effect of topical application of "T" fraction in patients with hemophilia or inhibitors of intrinsic thromboplastin. The easy availability of the "T" fraction together with its tolerance to heat sterilization lend itself to topical use. Application of the "T" fraction on a bleeding surface (immediately after the blood has been wiped off) in hemophiliacs initiates a normal local clotting process which goes quickly to completion yielding a firm adherent clot. Best results were obtained when applied on a dry wound surface. With external bleeding the topical "T" fraction reduced the number of trans-

Recalcified plasma hemophilia A.

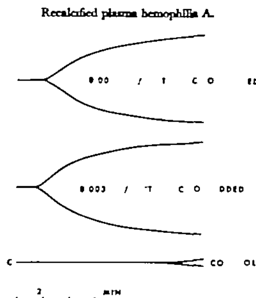


Fig 2. Induction of normal clot formation in recalcified hemophilia A-plasma upon addition of "T" fraction. A 0.0015 mg. "T" fraction/ml. plasma, almost complete normalization. B 0.003 mg "T" fraction/ml. plasma, complete normalization. C Untreated control. Thrombelastograms.

Table II
The use of topical sterile "T" fraction in hemophilia and circulating inhibitors of intrinsic thromboplastin.

Condition	How applied	Results
tooth extractions	gelfoam	excellent
cuts, lacerations	gelfoam	excellent
surgical procedures	gelfoam	good
epistaxis	packings soaked	fair
intestinal hemorrhages	drink (buffered)	inconclusive

fusions required or made them unnecessary Effectiveness in hemophilia was expected but the equally observed effectiveness in patients with circulating inhibitors was surprising Topically applied "T" fraction proved to be a very effective hemostatic agent in such patients involved in car accidents and bleeding continuously from multiple cuts and lacerations in spite of massive transfusions. A summary of clinical impressions obtained from about 25 cases with topical application of "T" fraction is given in table II

c) Neutralization of pathological inhibitors of intrinsic thromboplastin by "T" fraction in vitro The topical control of hemorrhages in patients with circulating inhibitors of intrinsic thromboplastin by

Table III

Correction of serum prothrombin times in patients with circulating inhibitors of intrinsic thromboplastin of various titers by "T" fraction in vitro (normal values 35–50 seconds)

Patient	Etiology	Without "T"	With "T"
M. R.	unknown	15	43
M. M.	AHG deficiency transfusions	17	42
R. C.	thrombopathia?	17	43
C. K.	unknown	19	35
No correction with 50–80% normal plasma			
G. F	lupus erythematosus	19	45
J. J.	PTC deficiency multiple transfusions	18	50
R. C.	unknown	17	36

Circulating inhibitor of intrinsic thromboplastin (low titer). Correction of deficient thrombin generation by "T"

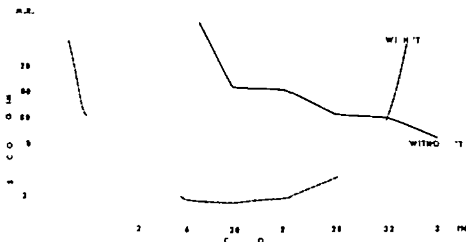


Fig. 2. Thrombin generation pattern with low titer circulating inhibitor of intrinsic thromboplastin before (black line) and after (dashed line) addition of "T" fraction. Abscissa: Incubation period after recalcification. Ordinate: Clotting time of fibrinogen solution to which an aliquot of the incubated recalcified citrated plasma after indicated incubation periods has been added

"T" fraction prompted investigations on its corrective action on their prothrombin consumption, thrombin generation, and thrombelastogram.

1) Prothrombin consumption. Table III shows the serum prothrombin time of patients with circulating inhibitors before and after addition of "T" fraction. In all instances the "T" fraction brought the short serum prothrombin times back to normal values. The effected correction is of particular interest in the last three

Circulating inhibitor of intrinsic thromboplastin (high titer) Correction of deficient thrombin generation.

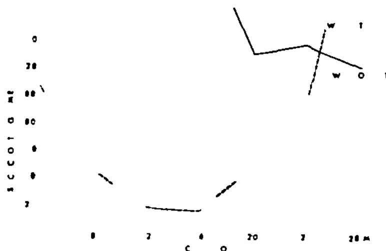


Fig 4 Patient J J Thrombin generation pattern with high titer circulating inhibitor of intrinsic thromboplastin before (black line) and after (dashed line) addition of "T" fraction. For further legend see fig 3.

patients of table III who exhibit a circulating inhibitor of high titer as shown by the inability of addition of 50–80 / of normal plasma to correct their serum prothrombin times.

2) Thrombin generation. The normalizing action of the "T" fraction on plasma with circulating inhibitors of intrinsic thromboplastin of various titers is further confirmed by the induced shift of the abnormal thrombin generation patterns into the normal range. Fig 3 reproduces the thrombin generation pattern of pa tient M R. *with a low titer circulating inhibitor* Note the poor thrombin generation in absence of the "T" fraction and the per fectly normal pattern in its presence This patient was transfusion resistent at the time of the test, but responded later to hydrocortisone treatment.

The following thrombin generation graphs were obtained from two patients (J J and R. C.) with high titer circulating inhibitors. Both did not respond clinically to even massive transfusions with out or with treatment with ACTH or hydrocortisone. Fig 4 shows the very poor thrombin generation of J J and effected the com plete correction after addition of "T" fraction.

The ineffectiveness of large amounts of normal plasma on prothrombin consumption of the other high titer patient R. C. is

Circulating inhibitor of intrinsic thromboplastin (high titer). Correction of deficient thrombin generation by "T"

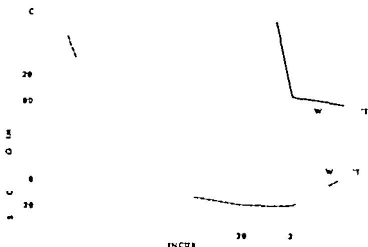


Fig. 5. Patient R. C. Thrombin generation pattern with high titer circulating inhibitor of intrinsic thromboplastin before (black line) and after (dashed line) addition of "T" fraction. For further legend see fig. 3.

Circulating inhibitor of intrinsic thromboplastin. Correction of thrombelastogram by "T"

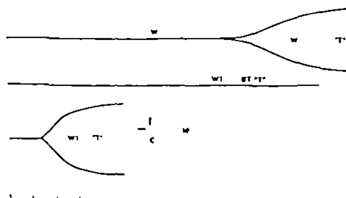


Fig. 6. Patient R. C. Thrombelastograms with high titer circulating inhibitor of intrinsic thromboplastin. Correction of plasma thrombelastogram by addition of "T" fraction.

demonstrated in table I. Here again, addition of "T" fraction normalizes the thrombin generation pattern as shown in fig. 5.

3) Thrombelastograms. The corrective effect of the "T" fraction on the greatly altered thrombelastogram of plasma from a patient

with circulating inhibitor of intrinsic thromboplastin reveals that the "T" fraction converts the abnormal kinetics of fibrin clot formation into nearly normal patterns. In fig 6 this is brought about by the thrombelastograms of patient R. C. This figure shows that with recalcified plasma of this patient no clot measurable by the thrombelastograph could be obtained within 240 minutes, whereas after addition of "T" fraction, within 29 minutes a normal clot started to form and went rapidly to completion. Other observations indicate that with an increased amount of "T" fraction added this time would have been shortened further

Discussion

In vitro correction of hemophilic blood clotting by small amounts of human urine as such was observed years ago (8 9) No efforts have been reported to isolate the responsible compound and to characterize some of its properties until more recently (1 2) The compound's ability to normalize hemophilia A clotting and to participate in the conversion of prothrombin into thrombin in the presence, but not absence of platelet factor 3 factor V and calcium (10) suggests some functional relationship to antihemophilic globulin. However heat stability excretion by hemophiliacs and the ability to neutralize with small amounts inhibitors of intrinsic thromboplastin seem to be specific properties of the "T" fraction. On the other hand, it does not possess some essential characteristics of tissue thromboplastin as shown by the heat stability of the antihemophilic effect and the requirement of platelet factor 3 for its full activity At least five times the amount needed for hemophilic blood is required to improve clotting of platelet poor plasma (2) One hundred times this amount does not reduce the clotting time of normal plasma below normal limits (11) It was also observed that methyl alcohol destroys its activity thoroughly (11) The toxicity is low 5 mg /kg rapidly intravenously injected into rabbits are tolerated producing a mild transitory thrombopenia and leucopenia similar to injections of macromolecules (2) All these observations do not yet permit a chemical or functional classification of the material.

It might be possible that the "T" fraction * consists of or con-

---

* It should be noted that it was recently proposed (13) to change the name "T" fraction to "P" fraction. The abbreviation "P" seemed to be more logical, since it was shown that the material is procoagulant rather than some type of thromboplastin.

tains a material which binds or inactivates inhibitors and related compounds. This assumption would also hold true for the corrective effect on hemophilic blood if one accepts that hemophilia A is caused by an inhibitor rather than by a deficiency a concept which was recently considerably substantiated by MARDERN (12)

From the practical point of view the procoagulant fraction from human urine deserves consideration as to its therapeutic potentialities, particularly in respect to circulating inhibitors of intrinsic thromboplastin.

Summary

A human urine fraction is described which in small quantities inactivates circulating inhibitors of intrinsic thromboplastin and normalizes hemophilic blood in vitro. The concentrated material exhibits good heat stability

Résumé

Les auteurs décrivent une fraction de l'urine humaine qui peut, en petites quantités, inactiver les inhibiteurs circulants de la thromboplastine intrinsèque et normaliser un sang hémophilique in vivo. Le matériel concentré présent une bonne résistance à la chaleur

Zusammenfassung

Es wird über eine Fraktion aus menschlichem Urin berichtet, die in kleinen Mengen die zirkulierenden Hemmstoffe von intrinsic Thromboplastin inaktiviert und hemophiles Blut in vitro normalisiert. Das konzentrierte Material weist eine gut Hitzestabilität auf.

References

1 VON KAULLA, K. N Methods for preparation of purified human thromboplastin and fibrinolysokinase from urine. Acta haemat. 16, 315 (1956).
2. VON KAULLA, K. N. Extraction and concentration of thromboplastic material from human urine. Proc. Soc. exp. Biol., N. Y 91 543 (1956) On purification and properties of urine thromboplastin. Proc. VIth Congr Int. Soc. Hemat. Boston 1956, p. 611 (Grune & Stratton, New York 1958)
3. VON KAULLA, K. N UND VON KAULLA, E. Spontane Schwankungen der Gerinnbarkeit menschlichen Blutes in vivo. Med. Welt. 36 1850 (1961).
4 BELL, W N AND ALTON, H. G A brain extract as substitut for platelet suspensions in the thromboplastin generation test. Nature 174 880 (1954)
5. VON KAULLA, K. N AND SWAN, H. Clotting deviations in man associated with open-heart surgery during hypothermia. J thorac. Surg. 36. 857 (1958)
6. VON KAULLA, K. N. Continuous automatic recording of fibrin formation and fibrinolysis A valuable tool for coagulation research. J Lab. clin. Med. 49 304 (1957).
7 MICHAELIS, L. Der Acetat-Veronal-Puffer Biochem. Z. 234 139 (1931)

8. G utike, W Studien über die Blutgerinnung mit besonderer Berücksichtigung der Hämophilie. I Einfluß des Harnes auf die Gerinnung hämophilen Blutes. Z. ges. exp. Med. 96. 512 (1935)
9 Tocantins, L. M. and Lindquist H. J : Thromboplastic activity of the urine. Proc. Soc. exp. Biol., N Y 65 44 (1947).
10. Caldwell, J von Kaulla, K. N von Kaulla, E. and Seegers, W H. Procoagulant material from human urine in prothrombin activation. Thromb. Diath. haem. (In press)
11 von Kaulla, K. N : (in preparation)
12. Mammen E. The coagulation defect in hemophilia A caused by an inhibitor Paper read t the XI th Annual Symposium on Blood, Detroit, January 1963.
13. Caldwell, J von Kaulla, K. N von Kaulla, E. and Seegers, W H. Procoagulant material from human urine in prothrombin activation. Paper read t the XI th Annual Symposium on Blood, Detroit, January 1963.

Authors' address. Dr K. N. von Kaulla and E. von Kaulla, Dept. of Medicine, Univ. of Colorado, 4200 East 9th Avenue, *Denver* 20, Colo. (USA).

Acta haemat. 30: 35–41 (1963).

Department of Medicine, Laboratory of Coagulation and Proteolysis, University of Louvain

Turnover of Prothrombin and Factors VII, IX and X in States of Hypocoagulability

By J. Hellemans*, R. De Vreker, M. Vorlat and M. Verstraete

It is remarkable that the disappearance rate of clotting factors is more rapid than that of most other plasma proteins. The half life of serum albumin is between 12 and 20 days (1–10) and even longer for gamma globulins (8–15). The half-life of fibrinogen however is 2.7–6.7 days (16–22) and of prothrombin 48–72 hours (23). The thrombocytes disappear also very rapidly from the peripheral circulation (life span = 10 days or less) (20, 23) in contrast to erythrocytes. We have established the half life and disappearance rate of prothrombin and factors VII, IX and X in a series of dogs after administration of a completely synthesis blocking dose of 3 different coumarin drugs. The results suggest that all coumarin drugs have the same rapidity of action after maximal dose administration (24).

It has been suggested that due to continuous coagulation a fibrin film covers the vascular endothelium (25–26) and this might be relevant to the maintenance of capillary permeability and prevention of haemorrhage. Several investigators have suggested that there might be a relationship between the rapid turnover of clotting factors and continuous haemostasis in vivo (17, 20, 27–32). The different arguments supporting this theory have been critically reviewed by Hjort and Hasselback (23) and they have concluded that much of the evidence is conflicting.

It has been demonstrated that even small modifications of molecules decrease their half life (7). Carter has suggested that similar structural changes occur in dogs during coumarin treat

* Aspirant of the N. F. W. O.

ment (32) The purpose of these investigations was to know if the half life of the various coumarin sensitive clotting factors was different before exposure of the dogs to coumarin derivatives (normal starting level of coagulation factors) and during coumarin administration (reduced starting level of coagulation factors)

Methods

The same methods as described before (33) were used.

A series of 5 dogs were given massive loading and maintenance doses of phenprocoumon (dogs 4 10, 11 and 12) or ethyl-biscoumacetate (dog 17). The half-life of the various coumarin sensitive clotting factors was followed as previously described (33) After 4 to 5 days 30 mg vitamin K_4 was administered intravenously while the same maintenance dose of the coumarin drug was continued. The first of the second series of blood samples was taken *9 hours after vitamin* K_1 *administration. The half-life of the* various clotting factors was determined again.

Results

The results are summarised in table I The upper part of the table corresponds to the turnover determined with normal (100 %) starting levels of the clotting factors concerned and the middle part to turnover studies assessed with reduced starting levels. More

Table I

Half-life in hours of different clotting factors, determined after administration of massive doses phenprocoumon (experiments 4 10, 11 and 12) or ethyl-biscoumacetate (experiment 17) on normal dogs (upper third) and in the same dogs, maintained in a state of decreased coagulation (second third).

	Exp.	F VII	F X	F IX	Prothr	F VII plus X	F II plus X	P and P [illegible]
A)	4	–	16.4	13.9	41.9	8.4	30.9	11.6
	10	7 46	22.5	11.5	64.2	14.8	46.5	20.4
	11	4 4	15.8	–	41 4	8.3	33.0	10.8
	12	5.91	17 4	16.9	48.0	9.8	36.7	15.4
	17		14 6		36.6	–	31.0	–
B)	4	–	16.4	17 7	38.6	11.9	33.9	15.1
	10	5.4	17 7	11.2	36.6	9.7	35.4	15.5
	11	6.3	14.8	–	31 1	9.6	32.6	14.3
	12	4.0	12.6	15.9	32.5	10.0	35.9	9.5
	17	–	14.0	–	32.2	–	32.9	
mean of A		5.9	17.3	14.0	46.4	10.6	35.6	14 4
mean of B		5.2	15.1	14.9	34.4	10.3	34.2	13.6

The low activity of the factor concerned, t the beginning of the experiment B did not allow a valuable measurement of the half-life.

Konakion, made available through the courtesy of Dr F VANDENDAEL, Roche Company Brussels.

Table II

Values of the different assays determined at the beginning and end of the two series of experiments in the different dogs. The (A) experiments were started in normal dogs, the (B) experiments in the same dogs with an induced state of decreased coagulation.

Experiment			Quick-time	P-and P test %	F VII %	F VII plus X %	F X %	Prothr %	F IX %	F II plus X %
4	(A)	Beginning	6'6	115	105	100	90	105	112	110
		End	1'27	5	<1	<1	<1	11,8	<1	6,4
	(B)	Beginning	9'3'	26	10 4	36,8	40	36,5	49	31 4
		End	1'7	<5	<1	<1	<1	<5	1	3,5
10	(A)	Beginning	7'6	98	77	200	79	85	91	82
		End	20'9	8,1	<1	7,5	4,6	32	1,6	20
	(B)	Beginning	8'3	36	16	40	37	33	43	33
		End	1'17	<5	<1	5 4	1,1	5,4	<1	5,4
11	(A)	Beginning	7'3	94	77	80	51	100	82	84
		End	24'8	<5	<1	4	1,8	20,5	2	12
	(B)	Beginning	8 4	35,5	35,5	47	40	42,6	36	34 7
		End	1'09	<5	<1	2,5	1,3	5,6	3	6
12	(A)	Beginning	7'3	86	50	125	50	75	105	68
		End	20'7	7,8	<1	5,4	1,8	18	3	12,8
	(B)	Beginning	8'8	60	8,1	52,7	85	32,3	36	35
		End	2'15	<5	<1	<1	6,8	<5	1,5	10,5
17	(A)	Beginning	7	100	65	88	74	110	92	90
		End	38'7	<5	<1	2,7	0,9	16,5	5	11,5
	(B)	Beginning	10'3	19	6,7	19	10	35	20,5	21
		End	54'3	<5	<1	2,5	0,6	13,8	5	9

details on the impaired coagulation of the dogs at the beginning of the second part of the experiment are given in table II where the values obtained at the beginning and end of each experiment are reported for the several dogs.

In only one dog (dog 12) is the starting level of the various clotting factors over 50 % of normal. All end values in the second half (i. e. after vitamin K_1 administration) of the experiments are even lower than the corresponding values in the first half of the experiment. A state of hypocoagulability was therefore obtained at the beginning of the second half of the experiments and it was presumed that the postulated continuous in vivo coagulation would therefore be retarded compared to the normal coagulation state.

As can be seen in the lower part of table I no significant difference was found in the half life determinations in both parts of the experiment. If the logarithm of the disappearance is plotted against time, a straight line is obtained for all clotting factors. As the slope of the disappearance for prothrombin remains constant even when

the 3 other clotting factors are very markedly decreased and bleeding occurs, additional evidence is obtained that prothombin disappears from the circulation at constant rate independently of the other three clotting factors

Discussion

The hypothesis of continuous intravascular coagulation has frequently been proposed (17 20 27 32) but so far no evidence has been offered that the turnover of clotting factors was slower in patients with permanent and severe blood clotting disorders. ADELSON has even found that the half life of fibrinogen does not change during dicoumarol administration (20) Transfusion experiments in patients with a congenital deficiency of a given clotting factor give half-life determinations equal or even shorter than those obtained in normal persons during coumarin administration (34 35 36) After blocking of the liver synthesis by large doses of Warfarin, in a patient with severe haemophilia A, HJORT et al. could prove that the turnover of factor VII was normal. It was probably also normal for prothrombin and factor IX (37) In a similar experimental procedure, the administration of heparin could not change the disappearance rate of proconvertin and of prothrombin (38)

The finding of similar half lives in dogs with normal coagulation or a state of hypocoagulation suggests that no changes in the molecular structure of the concerned coagulation factors occurred after the first poisoning of the dogs with huge doses of coumarin drugs.

It has to be mentioned that HOAG (39) found a higher disappearance rate of factor VII concentrate in patients with a coagulation defect (*i. e. congenital hypoproconvertinemia) compared to* normal individuals.

An important difference between both groups is however that one is producing factor VII and the other is not. The possibility remains that in one experiment the half life of factor VII concentrate alone is determined and in the second experiment on normal patients, the combined half-life of the concentrate and the endogenous production of factor VII is measured this could explain the difference between the two experiments.

Summary

The investigations were based on the working hypothesis that structural changes of clotting factors might be induced during coumarin administration and shorten their half-life. The results indicate that the half-life of coumarin sensitive clotting factors in dogs before and during exposure to coumarin derivatives are similar in both states.

Résumé

Les recherches sont basées sur une hypothèse de travail selon laquelle le traitement par al coumarine produit des modifications de la structure des facteurs de la coagulation, qui raccourcissent leur durée de vie. Les résultats démontrent que des facteurs sensibles à la coumarine du chien ont une même durée de vie avant et près le traitement à la coumarine.

Zusammenfassung

Die Untersuchungen basieren auf der Arbeitshypothese, daß bei Cumarin-Behandlung Strukturveränderungen der Gerinnungsfaktoren auftreten, die ihre Lebensdauer verkürzen. Die Ergebnisse zeigen, dass Cumarin-empfindliche Gerinnungsfaktoren beim Hund vor und während Cumarin-Zufuhr die gleiche Lebensdauer aufweisen.

References

1. Armstrong, S. H. Kukral, J Hershman J; Mac Leod, K. Wolter, J and Bronsky D. The persistance in the blood of the radioactive label of albumins, gamma globulins and globulins of intermediate mobility II. Comparison of gamma globulins labeled with S^{35} and I^{131} in the same subjects. J Lab. clin. Med. 45. 51–60 (1955).
2. Armstrong, S. H. MacLeod, K. Wolter, J and Kukral, J The persistance in the blood of the radioactive label of albumins, gamma globulins and globulins of intermediate mobility studied with S^{35} and paper electrophoresis methods and preliminary results. J Lab. clin. Med. 43. 918–937 (1954).
3. Armstrong, S. H.; Kukral, J; Hershman, J McLeod, K. and Wolter, J Comparison of the persistance in the blood of gamma globulins labeled with S^{35} and I^{131} in the same subjects. J. Lab. clin. Med. 44 762 765 (1954).
4. Berson, S. A.; Yalow R. A.; Schreiber, S S. and Post J Tracer experiments with I^{131} human serum albumin distribution and degradation studies. J clin. Invest. 32. 746–768 (1953)
5. Margen, S. and Tarver, H. Comparative studies on the turnover of serum albumin in normal and human subjects. J clin. Invest. 35 1161 1172 (1956)
6. Weissman, S. Tumulty D. and Bacchus, H. Use of precursor product relationship in determining serum albumin half-life. J Lab. clin. Med. 57 136–146 (1961)
7. Mc Farlane, A. S. Labelling plasma proteins with radioactive iodine. Biochem. J 62: 134–143 (1956).
8. Volwiler, W; Goldsworthy P D. Mac Martin, M. P; Wood, P A. Mackay J R.; Fremont-Smith, K. and Shock, D. F Turnover rates of various plasma proteins in normal and cirrhotic humans using biosynthetic methods. J clin. Invest. 33. 970–970 (1954).
9. Volwiler, W; Goldsworthy P D; Mac Martin, M. P Wood, P A.; Macka I. R. and Fremont-Smith, K. Biosynthetic determination with radioactive sulfur of turnover rates of various plasma proteins in normal and cirrhotic man. J clin. Invest. 34 1126–1146 (1955).

10. Volwiler, W. Freemont-Smith, K. and Mac Martin, M. P.: Turnover rates of serum proteins in cirrhotic man. J. clin. Invest. 31 668 (1952).
11 Gitlin, D. Janeway C. A. and Farr, L. E. Studies on the metabolism of plasma proteins in the nephrotic syndrome. I Albumin, γ-globulin and iron-binding globulin. J. clin. Invest. 35, 44–53 (1956).
12. Gitlin, D. Distribution dynamics of circulating and extravascular I[131] plasma proteins. Ann. N. Y. Acad. Sci. 70 122 136 (1957).
13. Bruton, O. C. Agammaglobulinemia. Pediatrics 9: 772 778 (1952).
14. Bruton, O. C. Apt L.; Gitlin, D and Janeway C. A. Absence of serum gamma globulins. Amer. J. Dis. Child. 84 632–636 (1952)
15. Martin, C. M. Gordon R. S. and Mc Cullough, N. B. *Acquired hypogammaglobulinemia in an adult.* Report of a case, with clinical and experimental studies. New Engl. J. Med. 254 449–456 (1956)
16. Gerber, K. and Maurer, W. Messungen zur Lebensdauer von Fibrinogen beim Menschen und beim Kaninchen. Biochem. Z. 328. 522–533 (1957).
17 Madden, R. E. and Gould, R. G. Turnover rate of plasma fibrinogen. Fed. Proc. 11 252–253 (1952).
18. Christensen, L. K. The turnover of plasma fibrinogen. Acta med. scand. 162: 407 414 (1958)
19 Hammond, J. D. S. and Verel, D. Observations on the distribution and biological half-life of human fibrinogen. Brit. J. Haemat. 5. 431–438 (1959)
20. Adelson, E. Rheingold, J. J. Parker, O. Buenaventura, A. and Crosby W. H. Platelet and fibrinogen survival in normal and abnormal states of coagulation. Blood 17 267 281 (1961)
21 Gitlin D. and Borges, W. H. Studies on the metabolism of fibrinogen in two patients with congenital afibrinogenemia. Blood 8. 679–686 (1953)
22. Ingram, G. I. C. Prockop, J. L. and Valley L. Survival in an afibrinogenaemic subject of fibrinogen from "time-expired" blood. Lancet 135–137 (1960)
23. Hjort P. F. and Hammerbach, R. A critical review of the evidence for continuous hemostasis in vivo. Thromb. Diath. haem. 6. 580 (1961)
24. Vermraete, M.; Hellemans, J. Vermylen C. and Verlat M. On the validity of the concept of slowly and rapidly acting coumarin drugs (to be published)
25. Roos, J. Blood coagulation as a continuous process. Thromb. Diath. haemorrh. 1 471–481 (1957)
26. Leveen, C. L. Electron microscopy of atheroma. Lancet i. 105 (1950)
27 Roos, J. Blood coagulation as continuous process II The turnover rate of proconvertin. Acta med. scand. 168. 477 (1960)
28. Alexander, B. Coagulation hemorrhage and thrombosis. New Engl. J. Med. 252 432–442, 484–494 526 (1955).
29 Stephanini, M. Mechanism of blood coagulation in normal and pathological conditions. Amer. J. Med. 14 64–86 (1953)
30. Jensen, H. Dynamic concept of fibrin formation and lysis in relation to hemorrhage (capillary permeability) and to thrombosis. Exp. Med. Surg. 14 189 (1956).
31 Laki, K. The clotting of fibrinogen. Blood 8. 845 (1953)
32. Gaertner, J. R. Nord Schow C. V.; Warner, E. and Lund, A. T. The in vivo synthesis of defective prothrombin molecules. Thromb. Diath. haemorrh. 5. 598–599 (1961)
33 Hellemans, J. Verlat M. and Vermraete, M. Survival time of prothrombin and factors VII IX, and X after completely synthesis blocking doses of coumarin derivatives. Brit. J. Haemat. (1963)
34 Loeliger, E. A. d. Esch, B. Clifton F. J. and Booij H. L. On the metabolism of the coagulation factor VII Proc. europ. Soc. Haemat. p. 223, 1959

35. Loeliger, E. A. and Hensen, A. Substitution therapy in haemophilia B. Thromb. Diath. haemorrh. 6. 391 (1961).
36. Borchgrevink, C. F., Egeberg, O., Pool, J. G., Skulason, T., Stormorken, H. and Waaler, B. A study of a case of congenital hypoprothrombinaemia. Brit. J. Haemat. 5. 294–301 (1959).
37. Hjort, P. F., Egeberg, A. and Mikkelsen, S. Turnover of prothrombin, factor VII and factor IX in a patient with hemophilia A. Scand. J. clin. Lab. Invest. 13. 668 (1961).
38. Hasselbach, R. and Hjort, P. F. Effect of heparin on in vivo turnover of clotting factors. J. appl. Physiol. 15. 945 (1960).
39. Hoag, M. S., Aggeler, P. M. and Fowell, A. H. Disappearance rate of concentrated proconvertin extracts in congenital and acquired hypoproconvertinemia. J. clin. Invest. 39. 554–563 (1961).

Authors' address: Drs. J. Hellemans, R. De Vreker, M. Verlat and M. Verstraete, Dept. of Medicine University of *Louvain* (Belgium).

Acta haemat. 30 42–64 (1963)

Aus der Medizinischen Klinik der Universität Pavia (Direktor: Prof. P Introzzi)

Lebendbeobachtungen über Bildung und Schicksal des Rabl'schen Polarfeldes in erythroblastischen Karyokinesen

Phasenkontrast-mikrokinematographische Untersuchungen

Von E. G Rondanelli P Gorini D Pecorari und E. Magliulo*

Bereits im Jahre 1885 hat Carl Rabl (8) in seiner klassischen Arbeit über die Mitose der Epithelialzellen von Amphibien beobachtet, daß im prophasischen Knäuel und in der Knäuelphase der Kernrekonstruktion die chromatischen Fäden eine bestimmte polare Orientierung haben.

Er schrieb: „Es ist gewiß kein Spiel des Zufalls, daß junge Tochterknäuel den Anfangsknäueln des Mutterkerns in ihrem Bau so außerordentlich ähnlich sehen. So wie sich ein Kern zur Theilung anschickt, oder aus einer Theilung hervortritt, läßt er ganz deutlich eine Polseite und eine Gegenpolseite erkennen, und an der Polseite selbst wieder eine enger begrenzte Stelle, das Polarfeld. Die einzelnen Regionen werden durch den Verlauf der Faden charakterisiert. Diese laufen von der Gegenpolseite aus, ziehen nach der Polseite und ins Polfeld, biegen hier schlingenförmig um und kehren wieder zur Gegenpolseite zurück» (8, S. 322). Das Polarfeld, mit anderen Worten, wäre eine bestimmte, aber keineswegs scharf begrenzte Stelle, die durch den Verlauf der Fäden besonders charakterisiert wird (8, S. 266).

Gemäß den Beschreibungen Rabls verläuft die Mehrheit der Fäden im prophasischen Kern in der Nähe der Zelloberfläche, und nur ein kleiner Teil geht durch das Kerninnere. Die Winkel, die diese zum Polarfeld bilden, werden primäre Winkel oder polare Winkel genannt. Die zahlreichen unregelmäßigen Krümmungen und Biegungen, *die die Fäden auf ihrem Verlauf aufweisen, heißen sekundäre Winkel. Die* primären Winkel sind konstant und als solche erkennbar während des ganzen Mitoseprozesses. Sie umgeben in der Prophase das Polarfeld, begrenzen in der Metaphase das zentrale achromatische Feld des Mutterkerns, ergeben in der Anaphase die primären Winkel der Tochterchromosomen, welche dann ihrerseits das zentrale achromatische Feld der Tochterkerne begrenzen und später das Polarfeld der telophasischen Knäuel der Tochterzellen zustande bringen. Es ist voraus-zusehen, daß im interkinetischen Kern eine Spur der polaren Orientierung der Fäden zurückbleibt, welche jedesmal, wenn die Zelle in Mitose eintritt, wieder deutlich erscheint.

* Services de Biologie Euratom.

Erst vor kurzem ist es PALUMBO (6) gelungen, in interkinetischen Kernen verschiedener Zelltypen die Existenz einer achromatischen Zone der Kernoberfläche von ihm «cariocentro» genannt, zu beweisen. Diese erscheint im allgemeinen als schmales Streifchen, umgeben von feinen Feulgen-positiven Körnchen (deren Zahl der Chromosomenzahl der untersuchten Zelltypen entspricht) mittels dünner Fäden mit den interkinetischen chromatinischen Strukturen verbunden. Außer an fixierten und gefärbten Präparaten ist eine solche Zone auch in interkinetischen Kernen von phasenoptisch untersuchten, in vitro überlebenden Zellen sichtbar in der Form einer unregelmäßigen, stark verlängerten Ellipse begrenzt von einer Umrißlinie mit deutlicher Seriereihung von stärker kontrastierten Teilchen, an denen die endonukleären chromatinischen Bildungen verankert sind» Nach PALUMBO, der im großen und ganzen mit der RABLschen Hypothese übereinstimmt, stammt das «cariocentro» vom Polarfeld der telophasischen Figur und von diesem stammt wiederum höchstwahrscheinlich das prophasische Polarfeld und dann das zentrale achromatische Feld des metaphasischen Sterns ab.

Neuester Entstehung ist die Hypothese von H. UND R. LETTRÉ (3, 4, 5) über die Beständigkeit der chromosomalen Spindelfasern während der Interkinese. Nach diesen Autoren geben diejenigen Fasern, welche das Centriolum mit den Chromosomen verbinden, den gleichen Verdoppelungserscheinungen entgegen wie diese letzteren und würden so für eine konstante Verbindung zwischen Polarzentren und Chromosomen sorgen. Die Kontraktion dieser immer bestehenden Fasern, im Fall eines extranukleären Centriolums, würde die Chromosomen verleiten mit ihren Zentromeren gegen die Kernmembran zu stoßen, die sogar dadurch deformiert werden könnte. Das Bestehen solcher Verbindungen, welche offensichtlich durch Löcher (wie sie übrigens am Elektronenmikroskop beobachtet wurden) der Kernmembran dringen, könnte uns die Begründung für die Wanderbewegungen der Chromosomen im Innern des prophasischen Kerns liefern. In der späten Telophase würden diese Fasern ihren Spannungszustand verlieren, aber dank ihrer Beständigkeit wären sie verantwortlich für die Polarisierungserscheinungen von RABL, da die Chromosomen dann eine Orientierung annehmen, die während der Interkinese bis zur darauffolgenden Prophase erhalten bleiben sollte. Diese Vorgänge könnten in ähnlicher Weise auch in Zellen mit einem intranukleären Anziehungszentrum erfolgen. Auch in diesem Fall würden die RABLsche Orientierung und die gegen das Kernzentrum gerichteten Bewegungen der Chromosomen erklärt werden.

Mit der vorliegenden Studie haben wir uns vorgenommen, erythroblastische Zellen von Triton in Karyokinese zu studieren. Diese Zellen sind sehr günstig für die morphologische Untersuchung am lebenden Objekt hinsichtlich der Entstehung und Entwicklung des Polarfeldes während der Karyokinese und auch hinsichtlich dessen Schicksals in den interkinetischen Kernen der zwei Tochterzellen nach der Zellteilung. Zu diesem Zweck haben wir zuerst eine Reihe normaler Mitosen im Phasenkontrastmikroskop untersucht, anschließend haben wir die Analyse auf karyokinetische Prozesse ausgedehnt, welche doppelkernige Zellen ergeben hatten (9) und schließlich auch auf pathologische Mitosen, bei welchen man Kernrekonstruktionen aus metaphasischen Figuren, die in ihrer weiteren Entwicklung aufgehalten wurden, beobachtet hatte (11) Die hier

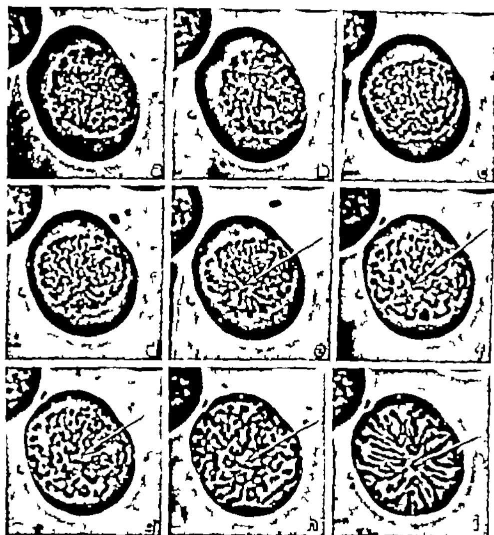


Abb 1 Erythroblastische Mitose von Triton. Prophase und Metaphase. Zeitfolge in Minuten der einzelnen Aufnahmen 0 b 12 19 d 27 38 f 42 g 44; h 46 i 57 *Gesamtvergrößerung ca. 800*

aufgeführten Beobachtungen sind mit Serienmikrophotographien aus mikrokinematographischen Aufnahmen dokumentiert

Material und Methoden

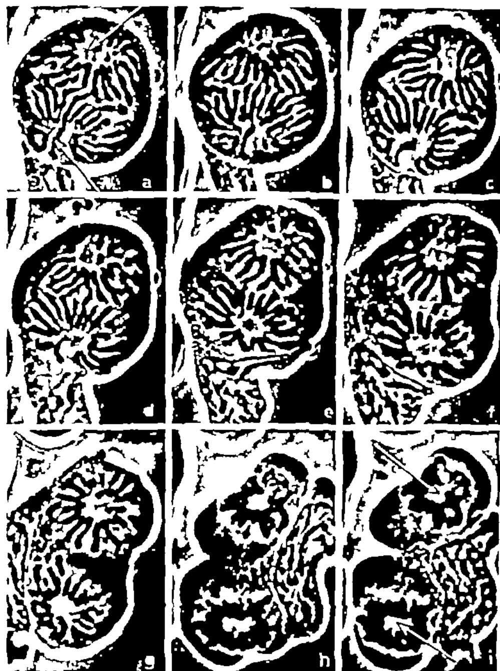
Die von uns untersuchten Zellen stammen aus Deckglaskulturen des peripheren Blutes von Triton (Molge vulgaris L.) in erythroblastischer Regeneration (2, 10). Die Karyokinese der Abb. 8, die in einer metaphasischen Kernrekonstruktion endet, stammt aus einem mit Benzol behandelten Tier (11)

Die mikrokinematographischen Aufnahmen wurden mit einem Zeiß-Siemens-16-mm-Apparat ausgeführt. Mit einer thermostatischen Einrichtung wurde die Temperatur der Präparate während der Aufnahme konstant auf 26 °C gehalten. Okular 6,3 × Objektiv Zeiß-Neofluar Ph, Ölimmersion, 100 ×. Vergrößerungsfaktor unserer Apparatur ca. 0,5, endgültige Vergrößerung auf dem Film ca. 300 ×. Die Photographien wurden mit einer zusätzlichen Vergrößerung gedruckt, die in den Erläuterungen zu den einzelnen Abbildungen als Gesamtvergrößerung angegeben wird.

Ergebnisse

Normale Erythroblasten-Mitose

1. Von der Prophase zur Metaphase. Die ersten 5 Photogramme der Abb. 1 (a–e) zeigen den Verlauf der Prophase in einer Erythroblasten-Mitose. Im Stadium des dichten Knäuels ist es unmöglich, eine Orientierung der Chromosom-Fäden wahrzunehmen. Diese erscheinen fein verknüpft und ohne Ordnung ineinander geschlungen. In Abb. 1 d, wo die Chromosomen kürzer und dicker geworden sind, ist eine Andeutung von polarer Orientierung der Chromosomen zu sehen, während in Abb. 1 e, welche kurz vor der Auflösung der Kernmembran aufgenommen wurde, die polare Orientierung deutlich sichtbar ist. Im Kernzentrum erkennt man eine unregelmäßige Zone, die von keiner besonderen Struktur begrenzt wird, aber durch das periphere Zusammenlaufen einer gewissen Anzahl primärer Winkel von Chromosomen gekennzeichnet ist, was dem Polarfeld von Rabl entsprechen sollte. Die Abb. 1 f, g und h zeigen deutlich, wie das prophasische polare Feld sich während der Metakinese verwandelt, bis daraus das zentrale achromatische Feld des metaphasischen Sterns entsteht (Abb. 1 i). Nach dem Verschwinden der Kernmembran wird der Verkürzungs- und Verdichtungsprozeß der Chromosomen zunehmend deutlicher. Die sekundären Krümmungen der Arme werden immer spärlicher, bis sie schließlich ganz verschwinden. Die primären Winkel der Chromosom-Schleifen treten hingegen deutlicher hervor und verschieben sich in solcher Weise, daß sie sich regelmäßig um die Zone des Polarfeldes gliedern, während sich die Schenkel strahlenförmig anordnen, wobei ihre freien Enden gegen die Zellperipherie gerichtet sind. Das Polarfeld, anfänglich nur vom Zusammentreffen eines Teils der Primärwinkel der Chromosom-Schleifen bestimmt, verwandelt sich dann allmählich im Verlauf der Metakinese und wird zum Zentrum, gegen das alle Primärwinkel der Schleifen zusammenlaufen. So entsteht die typische Figur des metaphasischen Sterns.



[illegible] – Erythroblastische Mitose von Triton. Anaphase und Telophase. Zeitfolge in Minuten der einzelnen Aufnahmen 0 b 5 10 d 14 16 f 17 g 21 h 24 25. Gesamtvergrößerung ca. 1300

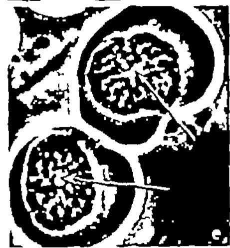
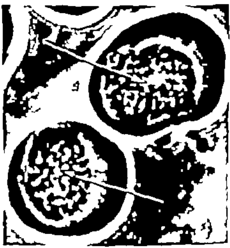
2 *Von der Anaphase zur Kernrekonstruktion* Die Abb 2 zeigt die Anaphase (a–g) und die Telophase (h, i) einer Erythroblasten-Mitose. Im Zentrum der Tochtersterne sind die zentralen achromatischen Felder gut sichtbar um die die Schenkel der Chromosom-Schleifen regelmäßig strahlenförmig angeordnet sind. Diese nähern sich einander nach und nach und werden kürzer und dicker Das zentrale achromatische Feld verkleinert sich immer mehr bis die chromatische Figur durch eine zunehmende Konglutination der Chromosomen das Bild einer kompakten, ringförmigen Masse von ungleicher Dichte und unregelmäßigen Grenzen annimmt, in wel cher keine Spur der Chromosomen mehr sichtbar ist, außer am Rand, aus welchem noch die freien, nicht zusammengeschmolzenen Enden von einigen Chromosomen auftauchen (Stadium des «kom pakten Sterns» 2)

Die Abb 3 zeigt die aufeinanderfolgenden Stadien der Kern rekonstruktion. In Abb 3a haben sich die beiden Tochterzellen eben gelöst. Ihre Kerne haben noch das oben beschriebene Aussehen von ringförmigen Massen mit unregelmäßigen Umrissen, ohne innere Struktur Die Chromosome sind nicht mehr sichtbar doch ragen noch einige freie Enden hervor Die Kernmembran ist erst in Abb. 3b sichtbar Zu diesem Zeitpunkt sind die Kernkonturen regelmäßiger geworden und im Innern erscheint eine Andeutung von Struktur Das Polarfeld ist immer im Kernzentrum als kleine, stark leuchtende Zone sichtbar rundlich im oberen, ovalförmig im unteren Kern.

Abb 3c zeigt die beiden Zellen in einem weit fortgeschrittenen Stadium der Rekonstruktion die Kernkonturen sind endgültig regelmäßig geworden, das Chromatin hat eine feinmaschige Struk tur angenommen, obwohl eine strahlenförmige Orientierung der dicksten Chromatin-Balken feststellbar ist, welche von der Peri pherie her gegen das Rabl'sche Feld hin zusammenlaufen. Das Polarfeld hat sich merklich verringert vor allem im unteren Kern. Oben, am Umriß des oberen Kerns ist eine kleine Delle sichtbar geblieben. Eine andere ist gegenüber sichtbar Es ist jedoch nicht festzustellen ob eine der beiden dem «Hilus» von Retzius ent spricht, oder ob es Einschnitte sind, die auf besondere Umstände der Kernrekonstruktion zurückzuführen sind, wie z.B Druck des Deckglases. Am Umriß des unteren Kerns ist andererseits keinerlei Einbuchtung sichtbar





Die Abb. 3d, e, f, nacheinander im Abstand einer Stunde aufgenommen, zeigen die weitere Wiederanordnung der endonukleären Strukturen. Die strahlenförmige Orientierung des Chromatin-Gerüstes ist immer weniger sichtbar, das RABLsche Feld schwindet noch mehr bis zur Unkenntlichkeit, wenigstens im unteren Kern. Im oberen bleibt hingegen eine kleine klare zentrale Zone von länglicher Form, deren größere Achse mit der des Kerns zusammenfällt und welche den interkinetischen Rest des RABLschen Feldes darstellt.

Selten haben wir Anaphase, Telophase und Kernrekonstruktion «im Profil» betrachten können. Ein solches Beispiel findet sich in Abb. 4. Während der Prophase, die keine Besonderheiten aufwies, war die polare Orientierung der Chromosomen nicht darzustellen. Sie war auch nach Auflösung der Kernmembran nicht zu erkennen; die Chromosomen befanden sich in der Zelle ohne jegliche Ordnung. Sie verschoben sich ständig, so daß sich das Aussehen der chromatischen Figur fortwährend änderte. Ob ein dem metaphasischen Stern entsprechendes Stadium auftrat, auch nur «im Profil» betrachtet, war nicht mit Sicherheit festzustellen. Vielmehr gewannen wir den Eindruck eines Überganges von einer eindeutig metakinetischen Anordnung zu der in Abb. 4a dargestellten Anordnung, wo auf beiden Seiten der Zelle die Bildung von zwei Polarfeldern deutlich erkennbar ist, und die Chromosomen mit ihren Schenkeln sich so zu gliedern beginnen, daß sie das Profil der beiden Tochtersterne entwerfen. Die Abb. 4b, c und d zeigen die polare Wanderung; bei einer solchen Anordnung der Zellen sind nur die Hälften der beiden Tochtersterne sichtbar. Die Abb. 4e und f entsprechen der Telophase. Die Erscheinung der telophasischen Pseudokonglutination der Chromosomen ist auch hier deutlich sichtbar. Die Delle, die die beiden chromatischen Figuren auf der der Teilungsebene der Zellen entgegengesetzten Seite aufweisen, stellt den Umriß um den hellen zentralen Raum – das telophasische Polarfeld – dar. Es folgt die Kernrekonstruktion (Abb. 4g, h, i), während der die chromatische Figur immer mehr an Volumen zunimmt. Dieses Anwachsen führt zum Verschwinden der oben beschriebenen Einbuchtung an der polaren Seite der beiden Tochter

Abb. 3. Dieselbe Zelle von Abb. 2. Kernrekonstruktion. Zeitfolge in Minuten der einzelnen Aufnahmen 27; b 29 39 d 60 120 f 180. Gesamtvergrößerung ca. 1500



Abb. 4. Erythroblastische Mitose von Triton. Anaphase und Telophase. Profil-Ansicht. Zeitfolge in Minuten der einzelnen Aufnahmen: 0 b 8 13 d 17 19 f 21 g 26 h 29 i 32. Gesamtvergrößerung ca. 800 ×

kerne, so daß im Stadium von Abb. 4i die polare und die gegenpolare Seite nicht zu unterscheiden sind. Die Chromatingerüste sind bei Beginn der Rekonstruktion (Abb. 4g) parallel zur kleineren Achse der Kerne gerichtet, was der strahlenförmigen Gliederung entspricht, die bei der Rekonstruktion der Kerne der Abb. 3 beobachtet wurde. Parallel zur fortschreitenden Kernrekonstruktion ver

schwindet diese Anordnung bis sie ganz unkenntlich wird wie im rechts unten gelegenen Kern der Abb 4 i.

Beispiele von abnormen Karyokinesen

1 Bildung von binukleären Zellen (Abb 5 6 7) Die 9 Photogramme der Abb 5 zeigen die Etappen der Anaphase bis zur Kernrekonstruktion in einer Erythroblasten Karyokinese, der keine Zellteilung folgt, so daß eine binukleäre Zelle zustande kommt, deren Entstehungsart schon anderswo eingehend besprochen wurde (9) Obschon es sich um eine atypische Mitose in bezug auf das Endresultat handelt, erscheint der Zyklus der chromatischen Figur ganz normal tatsächlich beobachtet man nacheinander die anaphasischen Tochtersterne, die telophasische Pseudokonglutination der Chromosome, das Wiedererscheinen der interkinetischen Chromatinstruktur Besonderes Interesse verdient aber die Entwicklung des Rablschen Polarfeldes In Abb 5 f ist es durch eine unregelmäßig ovale, helle Zone von beachtlichem Ausmaß im Kernzentrum dargestellt, die nach der größeren Kernachse ausgerichtet ist. Gleichzeitig mit dem Fortschreiten der Kernrekonstruktion schrumpft diese Zone zusammen aber die Reduktion erfolgt vor allem auf Kosten des kleineren Durchmessers, so daß sich das ursprüngliche Rablsche Feld (Abb 5 h und i) am Ende in ein ganz dünnes, helles Streifchen verwandelt hat, welches immer noch nach der größeren Achse des Kerns ausgerichtet bleibt Das Streifchen erscheint umgeben von einer dunklen ununterbrochenen Linie, längs der einige granuläre Verdickungen sichtbar sind Diese sind durch feine Fäden mit den Chromatin-Strängen des Kerngerüstes verbunden. Andererseits sieht man auch größere Chromatin Balken in Verbindung mit jener Linie, die die oben beschriebene helle zentrale Zone begrenzt. In Abb 6 werden die Photogramme h und i der Abb 5 bei stärkerer Vergrößerung wiedergegeben die Pfeile deuten die Reste der Polarfelder an Auch die Abb 7 zeigt eine atypische Mitose mit Entstehung einer doppelkernigen Zelle deren Genese schon anderswo beschrieben und diskutiert wurde (9) Hier ist zu betonen, daß auf ähnliche Weise wie bei der vorhergehenden Beobachtung die Entwicklung und der Zyklus der chromatischen Figur völlig normal ist, und vor allem, daß bei vollendeter Kernrekonstruktion im Zentrum der beiden Kerne eine klare Zone bleibt, welche zweifellos vom zentralen achromatischen Feld der Tochtersterne stammt. Im





Abb. 6. Die Photogramme h und i der Abb. 5 bei stärkerer Vergrößerung (ca. 1600 ×)

oberen Kern hat diese Zone eine längliche Form, ist nach der größeren Kernachse ausgerichtet und von einer dünnen, dunklen, ununterbrochenen Linie umrandet, in die einige der größten Chromatin Balken zusammenlaufen. Es fehlen hingegen die granulären Verdickungen, welche im vorhergehenden Beispiel sichtbar waren. Im unteren Kern wiederholen sich die Merkmale der hellen zentralen Zone des oberen Kerns, ausgenommen in der hier rundlichen Form.

2. *Rekonstruktion eines interkinetischen Kerns aus der metaphasischen Platte* (Abb 8). Die morphologische Untersuchung von Zellveränderungen durch Benzol, Gegenstand einer anderen Arbeit (11) gab uns Gelegenheit, die Entwicklung des zentralen achromatischen Feldes während einer pathologischen Karyokinese zu verfolgen. Diese wird durch die Wirkung des Gifts in der Metaphase aufgehalten, und endet in einer metaphasischen Kernrekonstruktion wahrscheinlich mit tetraploidem Chromosomensatz. Bis zur Bildung des metaphasischen Sterns (Abb 8a und b) wies die Karyokinese keine Besonderheiten auf. Anschließend, anstatt daß die polare Wanderung erfolgte, konglutinierten die Chromosome zunehmend und

Abb. 5. Bildung einer erythroblastischen zweikernigen Zelle von Triton. (Aus Rondanelli et al., 9). Zeitfolge in Minuten der einzelnen Aufnahmen: a 0; b 27; c 68; d 82; e 87; f 106; g 125; h 139; i 175. Gesamtvergrößerung ca. 900 ×



Abb. 7. Bildung einer erythroblastischen zweikernigen Zelle von Triton. (Aus Rondanelli et al. 9) Zeitfolge in Minuten der einzelnen Aufnahmen 0 b 8 37 d 50 90 f 105 g 125 h 138 150. Gesamtvergrößerung ca. 750 ×

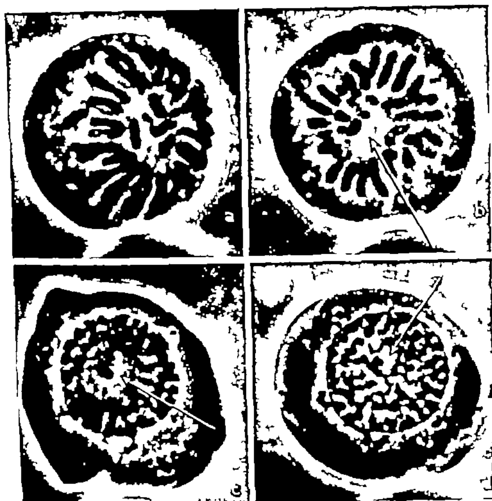


Abb. 8. Metaphasische Kernrekonstruktion bei Benzolvergiftung von Triton. (Aus Rosmarelli et al., 11) Zeitfolge in Minuten der einzelnen Aufnahmen 0 b 25 c 65; d 97 Gesamtvergrößerung ca. 1700 ×

bildeten eine einzige ringförmige Masse um die herum bald die Kernmembran entstand (Abb 8c) Im Zentrum des so rekonstruierten Kerns blieb der Rest des zentralen achromatischen Feldes als kleine, helle Zone von länglicher Form mit unregelmäßigen und undeutlichen Konturen gut sichtbar Eine halbe Stunde nach der Aufnahme der Abb. 8c kann die Rekonstruktion des Kerns als end gültig abgeschlossen betrachtet werden (Abb 8 d) Dennoch er

kennt man im Kernzentrum noch die vorher beschriebene kleine, helle Zone, obwohl sie nun eine rundliche Form angenommen hat und merklich kleiner geworden ist.

Diskussion

1 *In allen von uns während der Phase des dichten Knäuels untersuchten Mitosen war es nie möglich, eine polare Orientierung der Chromosomen und die Existenz einer Zone die als Polarfeld betrachtet werden könnte mit Sicherheit zu belegen* Das könnte damit zusammenhängen daß nicht gleichzeitig beide Zellseiten beobachtet werden können. Man kann sich auch denken daß die Orientierung der prophasischen Chromosomen in den Erythroblasten zunächst maskiert sei, und daß erst später im Stadium des lockeren Knäuels wegen der zunehmenden Verkürzung der einzelnen Chromosomen und der Abnahme der Zahl der sekundären Biegungen sich die polare Orientierung und das Polarfeld abzeichnen Auf Grund der Ergebnisse der vorliegenden Untersuchung gibt es jedoch keine gültigen Argumente, die diese Hypothese stützen. Es ist aber auch nicht anzunehmen daß die polare Orientierung der Chromosomen und das Polarfeld sich «de novo» während der späten Prophase bilden.

2 *Die polare Orientierung und das Polarfeld sind zu verschiedenen Zeitpunkten während der zweiten Hälfte der Prophase d h während der Phase des lockeren Knäules wahrzunehmen.* Auch JOLLY (2) und TÖRÖCK (14) haben bei ihren Untersuchungen von fixierten und gefärbten Erythroblasten von Triton das Polarfeld erst in karyokinetischen Figuren, die diesem Stadium entsprechen, beobachtet. Wir können ferner beifügen, daß während in gewissen Fällen die polare Orientierung schon einige Minuten vor der Auflösung der Kernmembran sichtbar ist, ist sie in anderen Fällen auch im Augenblick der Kernmembranauflösung (wenn überhaupt erst nachher) nur mit Mühe zu erkennen Auch dies könnte einerseits von der verschiedenen Orientierung des Kerns, andererseits von einer effektiven Veränderung im Auftreten der polaren Orientierung abhängen.

3. *In günstig ausgerichteten Zellen erscheint das Polarfeld in der späten Prophase und besonders während der Metakinese als rundlicher Raum, im Zentrum der chromatischen Figur gelegen, begrenzt durch die Primärwinkel der Chromosomschleifen* Wir können nicht mit Gewißheit behaupten, ob tatsächlich alle Primärwinkel um das Polarfeld herum zusammenlaufen, oder ob dieses um sich nur einen Teil davon versam

melt, weil es in diesem Stadium mit dem Phasenkontrastmikroskop unmöglich ist, die verschiedenen Chromosomen einzeln zu verfolgen und vor allem mit Genauigkeit alle Scheitel der dem Polarfeld entsprechenden Chromosomschleifen zu zählen. Nach Beginn der Metakinese, wenn die Chromosomen schon eine merkliche Verkürzung und Verdickung aufweisen und sich die Schenkel der Schleifen immer mehr gegen die Zellperipherie ausstrecken, befreit sich auch der Grundboden des Polarfeldes von den Fäden, die ihn vorher bedeckten und durch die gleichzeitige Verschiebung einiger Schleifen in exzentrischer Richtung verwandelt sich das Polarfeld in einen unregelmäßig zylindrischen Raum, der die chromatische Figur von einem Teil zum andern durchquert.

Bei sorgfältigem Drehen der Mikrometerschraube kann die Achse dieses Raumes, wenn sie der optischen Achse des Mikroskops entspricht, ohne Mühe von einer Seite zur anderen verfolgt werden. Beim selben Vorgehen bekommt man ferner den Eindruck, daß die ganze chromatische Figur im Achsenraume des hellen zentralen Raumes langsam zusammengedrückt wird. Folglich kann nun auch von der gegenpolaren Seite der Zelle das Polarfeld gesehen werden, da die distalen Portionen der Chromosomschleifen, die es anfänglich markierten, jetzt nicht mehr vorhanden sind. Übrigens hatte schon RABL behauptet, daß man zu einem gewissen Zeitpunkt nicht mehr von polarem und gegenpolarem Teil sprechen kann. Durch eine allmähliche Verschiebung der Chromosomschleifen verwandelt sich nämlich auch der gegenpolare Teil so, daß er dem ursprünglichen polaren Teil sehr ähnlich wird und von diesem Moment an weist der Kern zwei einander gegenüberliegende Polarfelder auf (vgl. RABL, 8, S. 242)

Denkt man nun an die räumliche Organisation des Kerns im Stadium des lockeren Knäuels oder der Metakinese, so versteht man leicht, daß in zahlreichen Fällen die oben beschriebenen Einzelheiten der Orientierung der Chromosome nicht gut zu beobachten sind. Es genügt in der Tat, daß die Achse des zentralen Raumes leicht schief zur optischen Achse des Mikroskops steht oder daß auch ein sehr geringer Druck einige Chromosomschleifen verschoben hat, um die Möglichkeit einzuschränken, solch delikate strukturelle Einzelheiten ausfindig zu machen.

4 *Am Ende der Metakinese wird die oben geschilderte helle Zone zum achromatischen Zentrum des metaphasischen Sterns* In den von uns beobachteten Erythroblasten Mitosen gelangt man tatsächlich zu einem Stadium, das einer sternförmigen Figur entspricht, deren Hauptebene (Teilungsebene) parallel zur Ebene des Objektträgers liegt. Das Sternzentrum wird durch einen rundlichen hellen Raum von relativ geringer Dichte gebildet begrenzt durch die Scheitel der Chromosomschleifen. Die sekundären Biegungen der Chromosomen sind nun fast vollständig verschwunden die Arme erstrecken sich strahlenförmig mit ihren freien Enden gegen die Peripherie, wie die Stäbe eines geöffneten Schirms. Erst in diesem Zeitpunkt kann mit Sicherheit behauptet werden, daß sich alle Scheitel der Chromo-

somschleifen rund um das zentrale achromatische Feld ansammeln, (außer natürlich in Fällen von anormalen Metaphasen, über die wir jedoch hier nicht berichten)

Mit unserer Beobachtungstechnik kommt man fast immer zu einem der oben beschriebenen Stadien welche auch immer die anfängliche Orientierung der Zelle gewesen sein mag wenn auch die Figur des metaphasischen Sterns nicht in allen Fällen so deutlich ist wie z.B in Abb 1 i Diese Erscheinung muß sehr wahrscheinlich auf besondere Umstände innerhalb der Zelle zurückgeführt werden welche, wenn auch nur leicht, zwischen Objektträger und Deckglas zusammengedrückt ist Deshalb ordnet sich die chromatische Figur so an, daß die Ebene in der sie sich vornehmlich entwickelt senkrecht zur Richtung der auf sie drückenden Kraft d h parallel zur Glasebene liegt Es ist tatsächlich vorstellbar daß der prophasische Kern mit seiner ungefähr sphärischen Form irgendwelche Lage in bezug auf die Gläser annehmen kann da sich aber seine Form allmählich ändert von einer kugelförmigen zu einer zusammengedrückten wird der Kern die oben geschilderte Stellung einnehmen

Wenn man wie JOLLY Präparate mit einem kleinen Abstand zwischen Objektträger und Deckglas verwendet wird der oben beschriebene metaphasische Stern nur selten sichtbar da die chromatische Figur nicht komprimiert wird Jedoch ist bei solchen Präparaten die Klarheit des Phasenkontrastbildes so sehr beeinträchtigt daß keine Mikrophotographien oder Mikrofilmaufnahmen angefertigt werden können

5 Im Moment der polaren Wanderung entstehen aus dem zentralen achromatischen Feld des Muttersterns die zentralen achromatischen Felder der Tochtersterne Hat sich nun ein typisch metaphasischer Stern gebildet, erhält man den Eindruck, daß, wenn sich die Tochterchromosomen endgültig getrennt haben, ein Tochterstern auf dem andern in Richtung auf einen Pol der Zelle hin gleitet, während sich der andere Tochterstern in entgegengesetzter Richtung verschiebt Die entsprechenden Stellungen der primären Winkel der Chromosomschleifen werden im allgemeinen beibehalten, so daß keine merkliche Deformation des zentralen achromatischen Feldes vorkommt, während die Schenkel der Chromosomschleifen verschiedene Verschiebungen ausführen können Fast durchwegs entsteht jedoch ein mehr oder weniger typischer Diaster mit sehr gut sichtbarem zentralem achromatischem Feld und strahlenförmig rund herum angeordneten Chromosomschenkeln Selten haben wir eine Rotation des metaphasischen Sterns festgestellt, eine Erscheinung, bei der dieser sich aus einer zur Ebene der Gläser parallelen Lage seitlich um 90° vor der polaren Wanderung dreht, und zwar so, daß auch die Tochtersterne diese Lage beibehalten

6 Der Anfang der Kernrekonstruktion wird durch eine Konglutination der einzelnen Chromosomen der Tochtersterne charakterisiert, so daß sich diese in ringförmige strukturlose Massen mit unregelmäßigen Rändern (Stadium des kompakten Sterns von JOLLY*) verwandeln* Es handelt sich um eine

schon an Erythroblasten von Triton beobachtete Erscheinung so von Töröck (14) und Jolly (2) dieser letztere hat ferner bemerkt, daß in normalen Mitosen die Teilung der Tochterzellen stets dann vorkommt, nachdem die chromatische Figur dieses Aussehen schon angenommen hat. Diese Pseudokonglutination der Chromosome bei der Bildung des kompakten Sterns wurde von uns auch in den mitotischen Prozessen aus welchen doppelkernige Zellen entstanden, wenn auch mit geringerer Deutlichkeit festgestellt. Es ist deshalb naheliegend, an eine Erscheinung zu denken, die dem Entwicklungszyklus der Chromosome eigen ist, der erste Schritt nämlich der Verwandlungen, die zur Rekonstruktion des interkinetischen Kerns führen und dies unabhängig von eventuellen Veränderungen der zytoplasmatischen Dynamik und der Mechanismen, welche die Anordnung des chromatischen Materials in den Tochterkernen regeln.

Während der gesamten Kernrekonstruktionsphase falls die Zelle günstig liegt ist innerhalb des Kerns der Rest des zentralen achromatischen Feldes des Tochtersterns sichtbar (telophasisches Polarfeld) Bei abgeschlossener Rekonstruktion ist dieses hingegen nur in wenigen Zellen sichtbar während es im allgemeinen nicht mit Sicherheit nachgewiesen werden kann. Während der Phase des kompakten Sterns» sieht der Rest des telophasischen Polarfeldes wie eine kleine helle Zone mit unregelmäßigem Rand aus, welche durch keine spezifische Struktur begrenzt wird. In einigen seltenen Fällen erscheint es später jedoch umgeben von einer dünnen Membran, wie in den Beispielen der doppelkernigen Zellen von Abb. 4 und 5 Während der Kern allmählich das balken netzförmige Aussehen des interkinetischen Zustandes annimmt, verkleinert sich in der Regel die helle Zone, bis sie meistens nicht mehr sicher festgestellt werden kann. Manchmal bleibt sie aber sichtbar wie beispielsweise in der oberen Zelle von Abb. 3c, und in der metaphasischen Rekonstruktion der Abb 8, auch wenn der nukleäre Rekonstruktionsvorgang als abgeschlossen bezeichnet werden darf

Man gewinnt tatsächlich den Eindruck, daß die ringförmige Masse, die den kompakten Stern bildete bei fortschreitender Kernrekonstruktion aufquillt, während der Rest des zentralen achromatischen Feldes des Tochtersterns sich langsam, während der Kern wieder sphärische Form annimmt, in einen mehr oder weniger großen Spalt verwandelt, der den Kern von einem Ende zum andern durchzieht. Man überzeugt sich am besten von dieser Erscheinung mittels direkter Beobachtung der Zelle in günstiger Lage, indem man diese langsam von oben nach unten und umgekehrt bei sorgfältigem Drehen der Mikrometerschraube beobachtet. Dieser Spalt bildet sich allmählich mit

zunehmender Verwandlung der Kernstruktur zurück, [illegible]hne daß er jedoch zu existieren [illegible]ufhört. Es ist tatsächlich so, daß die mangelhaf[illegible] Besichtigung des telophasischen Polarfeldes, wie es in der Mehrzahl der Fälle [illegible]orkommt, von der Verdichtung des endonukleären Gerüstes [illegible]bhängig ist, welche es [illegible]on den anderen Maschen des Chromatin-Netzes nicht mehr unterscheiden läßt. Die Dauer der Sichtbarkeit des Polarfeldes hängt vermutlich von Faktoren [illegible]b, w[illegible]lche die [illegible]otale Kernrekonstruktion [illegible]erhindert oder [illegible]erzögert haben. So einmal der Druck, der das Annähern der Ränder des Polarfeldes verzögern kann, während er [illegible]ur geringe Wirkung [illegible]uf das Fortschreiten der inneren Gliederung der Chromatinstruk[illegible]uren hat. Es können so Bilder entstehen, beginnend von einem normal geformten Kern mit einem kleinen hellen Raum im Zentrum, der das Überbleibsel des Rabl'schen Feldes anzeigt, bis zu einem ringförmigen Kern, der die ursprüngliche Form des [illegible]elophasischen kompakten Sterns beibehalten hat.

8. Der Verlauf d[illegible] [illegible]polaren Orientierun[illegible] der Chromosomen während der Mitose ist ein [illegible] [illegible] [illegible]chen [illegible] [illegible] die gleichzeitige Entwicklung des achromatis[illegible] [illegible]n[illegible] [illegible]pa[illegible] [illegible](Spindelapparat). Mit dem Phasenkontrastmikrosk[illegible] [illegible] [illegible] [illegible] m[illegible]gl[illegible]h [illegible] die achromatischen Strukturen zu beobac[illegible] [illegible]n [illegible]ed[illegible] [illegible]ttet das Studium der Veränderungen der chro[illegible] [illegible] [illegible] [illegible] diesbezügliche Folgerungen. Das Er[illegible]sch[illegible] [illegible] [illegible] [illegible] pol[illegible]ren Or[illegible]ierung in den späten Prophase ist offen[illegible] [illegible] [illegible]h verbund[illegible]n ent[illegible] [illegible]d[illegible] mit dem vermutlichen Andauern der [illegible] [illegible]rung [illegible]eit der vorhergehenden Telophase durch die [illegible] [illegible]hind[illegible] [illegible]h bis zur neuen Prophase, oder mit dem [illegible] [illegible] [illegible] Orient[illegible]rung während der Prophase durch d[illegible] E[illegible] [illegible] [illegible] [illegible]entr[illegible]n. In beiden Fällen könnte die Er[illegible]scheinung [illegible] [illegible] [illegible] [illegible]klärt werden, als daß die Zentromeren der Chromosomen [illegible] [illegible] der Höhe der primären Winkel liegen) [illegible] [illegible]ndwie mit d[illegible] [illegible] [illegible]ngszentrum verbunden bleiben. Andererseits ist es [illegible] [illegible] [illegible] in unserem Fall, die Natur dieser Verbindungen ge[illegible] [illegible] zu [illegible]ild[illegible]rn, noch können wir das Anziehungszentrum mit Bezeichnungen belegen, die einem morphologischen Bild entsprechen (Centriolum, Centrosom, usw.). Man kann jedoch vermuten, daß die besondere Polarisierung der Chromosomen in der Prophase einer Orientierung um den sich bildenden Spindelapparat zugeschrieben werden kann, und dies in Übereinstimmung mit einigen Autoren, die die Möglichkeit hervorheben, daß die Bewegungen der Chro[illegible]somen schon vor der Auflösung der Kernmembran beginnen, u[illegible] [illegible] [illegible]3 die Chromosomen auch unter diesen Umständen unter der Kontrolle der Anziehungszentren stehen.

Fell und Hughes (1) haben tatsächlich beobachtet, daß die Chromosomen im Innern des prophasischen Kerns sich an die Kernmembra[illegible] [illegible]n Zonen gliedern, welche sich in der Nähe der [illegible]ußerhalb der Membran stehenden Anziehungszentren befinden. Schrade[illegible] [illegible]13) hat in Eiern [illegible]on Annelides während der ersten meiotischen Teilung beobacht[illegible]et, daß die Chromosomen im Kerninnern gleichzeitige Bewegungen zu denen

der Anziehungszentren ausführen, und daß anschließend die Wanderung zu den beiden Zellpolen erfolgt. Diese Untersuchungen zeigen, daß die Chromosomen die Auflösung der Kernmembran nicht abwarten müssen, um eine bestimmte Orientierung anzunehmen, und daß die Kernmembran selbst kein Hindernis für die Wirkung zwischen Anziehungszentren und Chromosomen darstellt.

Im Fall der von uns untersuchten Zellen ist es hingegen sehr wahrscheinlich, daß der Zentralapparat sich im Innern des interkinetischen und prophasischen Kerns befindet. Wenn im Erythroblast von Triton die endonukleäre Lage des Zentralapparats auch nicht direkt gezeigt werden kann, kann sie doch auf Grund indirekter Daten abgeleitet werden. In diesen Zellen wurde nämlich während der Interkinese und der Prophase das Vorkommen außerhalb der Kernmembran von einem oder allen konstitutiven Elementen des Zentralapparates nie beobachtet, im Gegensatz zu dem, was in anderen Zellen von Triton gesehen wurde (Granuloblast, Lymphoblast, Histiozyt usw.), bei denen der Zentralapparat immer außerhalb der Kernmembran mit allen seinen konstitutiven Elementen (Centriolum, Centrosom, mitochondriale Strahlung) (vgl. 12) gesehen wurde. Ferner kann die endonukleäre Lage der Anziehungszentren im Tritonerythroblast von den folgenden Daten im Vergleich mit anderen Zelltypen, bei denen die Anziehungszentren außerhalb des Kerns liegen, abgeleitet werden:

a) In Zellen mit extranukleärem Zentralapparat zeigen die Mitochondrien charakteristische strahlenförmige Hin- und Herbewegungen auf die zentrosomale Zone gerichtet. Dagegen erscheinen die Mitochondrien in den hier untersuchten Erythroblasten ohne irgendwelche Ordnung im Zytoplasma zerstreut und zeigen keine geordnete Bewegungen.

b) Der Kern der Erythroblasten stellt sich sphärisch dar, während die Zellen mit extranukleärem Zentralapparat meist eine kennzeichnende Einbuchtung des Kerns auf Höhe der zentrosomalen Zone aufweisen (7).

c) Der prophasische Kern der Erythroblasten weist vollständige, ruckartige Rotationsbewegungen um die eigene Achse auf, was in anderen Zelltypen, bei denen der Zentralapparat sicher extranukleär ist, nicht vorkommt; der Zentralapparat ist nämlich mit der Kernmembran verbunden und verhindert das Zustandekommen solcher Bewegungen (4, 5).

Es ist also zu vermuten, daß in den von uns untersuchten Zellen der Zentralapparat sich in der Gegend des Rablschen Polfeldes organisiert, und anschließend die Verwandlung des lockeren Knäuels in einem metaphasischen Stern sowie die Teilungsachse der Zelle bestimmt.

Nachdem die polare Wanderung und die Trennung der Tochterzellen erfolgt sind, erhält jede dieser beiden die Hälfte des ursprünglichen Zentralapparats, der sich in der Mitte der ringförmigen, von den Chromosomen gebildeten Masse befindet. Die beiden Anziehungszentren liegen innerhalb der entsprechenden achromatischen Felder und sind wahrscheinlich für das konzentrische Zusammenziehen der Chromosomschleifen rund um das achromatische Feld verantwortlich. Das Andauern der strahlenförmigen Orientierung des Chromatingerüstes nach dem Stadium des kompakten Sterns (vgl. z. B. Abb. 3c, d, e) bestätigt die Annahme, daß die Verbindungen der Scheitel der *Chromosomschleifen mit dem*

Anziehungszentrum immer noch erhalten sind und daß dieses im Polarfeld des sich rekonstruierenden Kerns liegt. Mit einer solchen Hypothese stimmen auch die morphologischen Befunde der Mitosen, die doppelkernige Elemente ergaben, überein.

Was die metaphasische Rekonstruktion von Abb 8 angeht, bemerken wir daß das Benzol eine Verletzung bewirkt hat, wodurch die polare Wanderung nicht erfolgen konnte. Jedoch erlaubt die Tatsache, daß die Kernrekonstruktion ohne eine Umorientierung der Chromosomen eintritt den Schluß daß die Verbindungen der Scheitel der Chromosomschleifen mit dem Anziehungszentrum auch in diesem Fall nicht unterbrochen werden

Schlußfolgerung

Zusammenfassend zeigen die hier beschriebenen Untersuchungen, daß im lebenden Erythroblast von Triton (Molge vulgaris L.) die polare Orientierung des RABLschen Feldes in günstig orientierten Zellen schon im Stadium des lockeren Knäuels nachweisbar ist. Aus dem prophasischen Polarfeld entsteht anschließend das zentrale achromatische Feld des metaphasischen Sterns. Aus diesem leiten sich die zentralen achromatischen Felder der Tochtersterne ab die sich in den hellen Spalten verwandeln die während der Kernrekonstruktion sichtbar sind. Dieser Spalt verschwindet meistens wieder und bei fortgeschrittener Rekonstruktion ist er nicht mehr mit Sicherheit nachweisbar Alle diese Übergänge und Veränderungen sind nur in gut orientierten Zellen sichtbar nämlich wenn ihr polarer Teil gegen die Frontlinse des Objektivs gerichtet ist. In anders orientierten Zellen entgehen uns viele dieser morphologischen Einzelheiten Andererseits stellt die «im Profil» angelegte Untersuchung der Karyokinese eine nützliche Ergänzung der frontalen Beobachtung dar besonders weil sie hilft, sich die Tridimensionalität der chromatischen Figur besser vorzustellen.

Was die mehr oder weniger gesicherte Identität der Überbleibsel der Polfelder wie sie in den interkinetischen Kernen der Tochterzellen sichtbar sind mit dem achromatischen Streifchen von PALUMBI 6) betrifft können wir ohne weiteres diese Möglichkeit annehmen. Es bekräftigen diese Behauptung vor allem die morphologischen Analogien, welche zwischen den Überbleibseln der polaren Felder die von uns in den Kernen der Tochterzellen beobachtet wurden und den von PALUMBI in Ruhekernen beschriebenen Struk

turen bestehen. Die vorliegenden Untersuchungen verbürgen also die Annahme, daß das von PALUMBI beschriebene »cariocentro« das interkinetische Äquivalent zum RABLschen Polarfeld der telophasischen Figur darstellt.

Zusammenfassung

An Hand von mikrokinematographischen Untersuchungen an lebenden Erythroblasten von Triton mit dem Phasenkontrastmikroskop beschreiben die Verfasser die Entstehung des RABLschen Polarfeldes während der Prophase, seine Umwandlungen während des ganzen Zellteilungsvorgangs und sein Schicksal in den Ruhekernen der Tochterelemente. Es wird die Auffassung vertreten, daß die neulich von PALUMBI in Ruhekernen beschriebene Struktur (achromatisches Streifchen, »cariocentro«) mit großer Wahrscheinlichkeit das Überbleibsel des RABLschen Polarfeldes der telophasischen Figur darstellt.

Summary

Cinemicrographic examination of living triton erythroblasts with the phase contrast microscope revealed the development of RABL polar field during the prophase its modifications throughout the whole mitotic process and its fa m the resting nuclei of the daughter cells. The view is put forward that the achromatic strip or carlocentro recently described by PALUMBI in resting nuclei is in all probability th remnan of the RABL polar field of the telophasic element.

Résumé

Les auteurs décrivent à l'aide d'études microcinématographiques d érythroblastes vivants du triton sous le microscope polarisant la formation du champ polaire de RABL pendant la prophase ses transformations pendant toute la division cellulaire et son destin dans les noyaux de repos des cellules-filles. Les auteurs sont d la que les struc tures des noyaux de repos, décrites dernièremen par PAL MBI (bandelette achromatique, cariocentro) représentent très probablement les restes du champ polaire de RABL de la télophase.

Literatur

1 FELL, H. B. AND HUGHES, A. F W Zit. 13.
2. JOLLY J Recherches expérimentales sur la division indirecte des globules rouges. Arch. Anat. micr 6 433–562 (1903/1904)
3. LETTRÉ, H. UND LETTRÉ, R. Persistenz der Chromosomen-Spindelfaser eine Arbeitshypothese zur Deutung der karyokinetischen Vorgänge. Naturwissenschaften 44 406 (1957)
4 LETTRÉ, H. ET LETTRÉ, R. U problème cytologique. La persistance des structures du fuseau dans l'intervalle des mitoses. Rev Hémat. 13 337–362 (1958).
5. LETTRÉ, H. ET LETTRÉ, R. Le mécanisme de la mitose discuté à la lumière de l'hypothèse de travail sur la persistance de la fibre fusoriale du chromosome; in Action antimitotique et caryoclasique des substances chimiques, pp. 25–40 (Editions du Centre National de la Recherche Scientifique, Paris 1960).

6. Palumbo, G. Sulla persistenza nel nucleo riposo di un campo polare residuo il carnocentro. Z. Ana 170 302 326 (1958
7 Policard, A. et Bessis, M. Étude microcinématographique et micro-électronique du centre cellulaire des leucocy es des vertèbres. Exp. Cell Res. 4 202 207 (1953).
8. Rabl, C. Über Zellteilung Morph. Jb. 10 214–328 (1885).
9 Rondanelli, E. G. Gorini, P Pecorari, D und Fiori, G. P Über die Bildung von doppelkernigen hämopoetischen Zellen phasenkontrastmikrokinematographische Untersuchungen. Z. Zellforsch. 49 668–676 (1959).
10. Rondanelli, E. G. Gorini, P and Pecorari, D. Method for testing the influence of drugs and physical agents upon mitosis. Nature 183 190 (1959)
11 Rondanelli, E. G. Gorini, P Pecorari, D.; Trotta, N et Colombi, R. Effets du benzène sur la mitose érythroblastique. Investigations à la microcinématographie en contraste de phase. Acta haemat. 26 281 302 (1961).
1. Rondanelli, E. G T otta, N. Scotti-Foglieni, C. et Vaccari, V La centrosphère d l'leucocy d Tri on. Étude en contrast d phase à l'état ivant. Nouv Rev franç Hema 1 55–64 (1961).
13. Schrader F Mitosis ..nd Ed. (Columbia Univ Press, New York 1953).
14 Torók, L. Die Theilung der rothen Blutzellen bei Amphibien. Arch. mikr. Anat. 1 603–613 1888

Adresse der Autoren Dr E. G Rondanelli, Dr P Gorini, Dr D. Pecorari und Dr E. Magliulo, Medizinische Un... Klinik, Pavia Italien).

Acta haemat. 30 65–87 (1963)

From the Department of Medicine, University of Freiburg i. Br
(Director Prof. L. Heilmeyer)

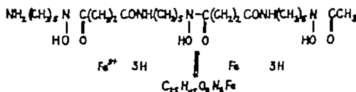
The Treatment of Haemochromatosis with Desferrioxamine

(With Plate I)

By Friedrich Wöhler

Hitherto venesection has had to be regarded as the method of choice for treating haemochromatosis. Now however we have available another means of withdrawing iron from the human organism. The great advantage of this new method over venesection is that it can also be employed in cases of transfusion haemosiderosis, haemolytic anaemia, and achrestic anaemia, as well as in the many different forms of secondary siderosis.

The method consists of administering hydroxamic acid compound, known as desferrioxamine B hydrochloride. Attempts to remove iron ions from the organism with the acid of chelating agents were undertaken as far back as 1952 (5, 6, 7 8) The drugs



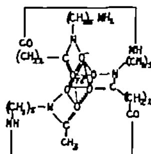


Fig 1

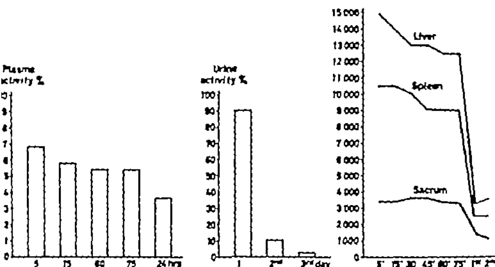


Fig. 2. Behaviour of radioactive ferrioxamine B in the organism (intravenous injection of 30 μc. ferrioxamine labelled with Fe^{59}).

used were dioxyethylglycine ethylenediaminetetraacetic acid and BAL. Recently use has been made of diethylenetriaminepentaacetic acid (DTPA) (4, 5). We have also employed this substance but it was not successful in long-term therapy because of toxic side effects.

Desferrioxamine is a naturally occurring substance which is formed from *Actinomycetes*. It belongs to the category of siderochromes which are able to form brownish red complexes with trivalent iron. The siderochromes can be divided into two main groups, the so-called sideromycins which are iron-containing antibiotics, and the sideramines, which possess growth-promoting properties and specifically antagonise the antibacterial effect of the sideromycins.

The desferrioxamine in which we are interested here is the iron-free parent substance of ferrioxamine, a member of the sideramine group. Desferrioxamine is a filamentous trihydroxamic acid derivate which forms an octahedral complex with an Fe^{+++} ion. The long chain of desferrioxamine coils itself round the iron atom so that latter is thoroughly embedded (fig. 1). 100 mg. desferrioxamine B hydrochloride is capable, theoretically, of binding 9.3 mg. Fe^{+++} ions. This complex composed of iron and hydroxamic acid has a molecular weight of 615 and can therefore pass very easily through the kidney. The stability constant of the Fe^{+++} complex is 10[illegible] and is thus somewhat higher than that of transferrin (10[illegible] and 10[illegible]) which possesses two binding centres. The stability constants for alkaline earths are lower than those for EDTA (for calcium 1.7 × 10[illegible] and for magnesium 2 × 10[illegible]). This also explains why desferrioxamine does not exert any untoward effects on the serum electrolytes even when administered for periods of more than one year.

Investigations to date have shown that other heavy metal ions, particularly trace elements, are not affected by desferrioxamine. Consequently it appears definite that the drug displays a selective effect on the iron proteins in the organism.

Ferrioxamine was prepared in a pure condition, defined and synthesised in the Research Laboratories of CIBA, Basle (1 2, 10)

Distribution and Excretion of Ferrioxamine

Originally we attempted to use ferrioxamine in the treatment of iron-deficiency anaemias. Despite the administration of extremely large doses, however neither the haemoglobin nor the erythrocyte level rose, but we were struck by a short lived rise in the serum iron. To obtain information about the metabolism of iron in the body radioactive ferrioxamine was administered intravenously As fig 2 shows, some 90 % of the labelled ferrioxamine was already

Table I

Behaviour of radioactive ferrioxamine in the organs of the rat (mean values of groups of 5 animals each) in terms of the time elapsing after injection of 15 µc. Fe^{59} (values expressed in % of the amount injected and calculated per g. of tissue).

Organs	after 5 min.	after 20 min.	after 24 hours
Liver	4.47	2.63	0.15
Spleen	2.82	1.56	1.30
Kidney	1.96	1.38	0.80
Heart	2.20	0.82	0.99
Lung	2.05	5.32	0.76
Bone marrow (thigh)	2.50	1.50	1.12
Thigh muscle	0.43	0.30	0.36
Thymus	2.49	2.10	2.00
Stomach	1.70	1.29	0.60
Duodenum	0.85	1.29	1.52
Small intestine	0.67	0.88	0.78
Large intestine	0.63	0.65	0.68
Abscess	1.50	1 40	1.88
Skin	0.22	0.30	0.15

excreted with the urine on the first day—a finding which, with slight deviations, was recorded time and again in repeated studies. By the third day only very small amounts of ferrioxamine could still be detected. In the serum, an activity of some 20 % was found 5 minutes after the injection of ferrioxamine, but this activity rapidly declined thereafter amounting to only about 2 % after 24 hours. After 72 hours no activity could be demonstrated any longer

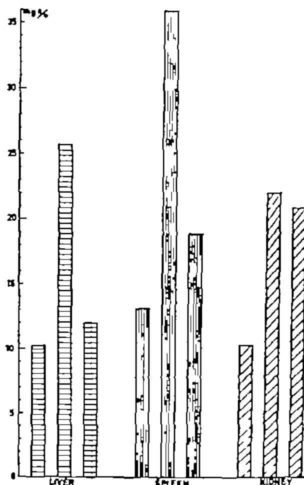


Fig. 5. Behaviour of organ iron in the rabbit following administration of ferrum vitis, as well as of ferrum vitis and desferrioxamine B. 1 = Normal animals 2 = 20 mg ferrum vitis i.v. 3 = 20 mg. ferrum vitis and infusion of desferrioxamine.

venous injection of 20 mg. ferrum vitis (ferric saccharate complex) the organ iron concentration rose. Subsequent infusion of 1 g desferrioxamine over a period of 4 hours led to an appreciable fall in the concentration of organ iron in the test animals as compared with the controls. The difference was least marked in the kidney because renal excretion was still not completed 12 hours after the infusion and a relatively large amount of iron was still present in the kidney (fig. 5)

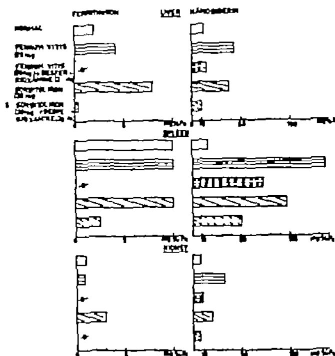
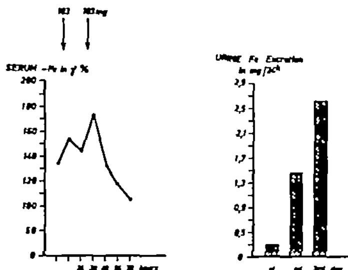


Fig. 4 Ferritin and haemosiderin iron in various organs of the rabbit following the administration of ferrum vitis (20 mg. i. v.), sorbitol iron (20 mg. i. v.) and desferrioxamine (2 g. i. m.).

Simultaneous determination of the ferritin iron and of the haemosiderin iron in these rabbit experiments revealed, as can be seen from fig. 4 that in the organs the ferritin iron, which is apparently more readily available, is released almost completely to the desferrioxamine. Following the administration of ferric saccharate complexes (ferrum vitis) or sorbitol iron, the concentration of ferritin and haemosiderin iron in the liver, spleen and kidney invariably rose. Even though, as exact quantitative determinations showed, the mass of the mobilized iron came from the haemosiderin, the ferritin iron was none the less almost completely eliminated. It may thus be concluded that both fractions of depot iron release their iron to desferrioxamine in the cells. Since ferritin can release its entire content of iron, the actual turnover of depot iron between ferritin and haemosiderin is probably also increased – that is to say the apoferritin that is produced will endeavour to take up the haemosiderin iron present, thus intensifying the breakdown of the

)



b)

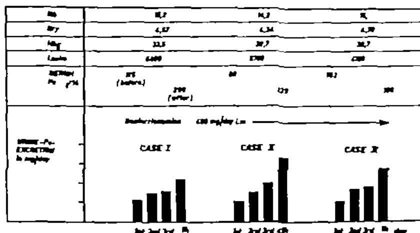


Fig. 5.) Serum iron level and urinary iron excretion following the administration of desferrioxamine in normal test subject. b) Urinary iron excretion following administration of desferrioxamine in 3 normal test subjects.

haemosiderin. Determination of the serum iron in the rabbit before and after the administration of desferrioxamine in these experiments clearly showed that the serum iron level rose during the mobilisation of the depot iron. This finding was considerably more marked in human beings.

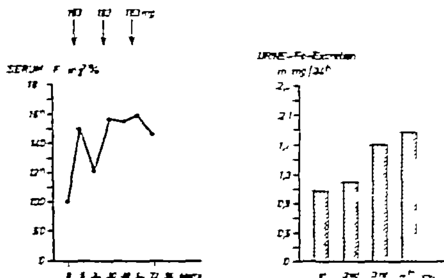


Fig. 6. Serum iron level and urinary iron excretion following the administration of desferrioxamine in a normal test subject (300 mg. Fe++ daily by mouth).

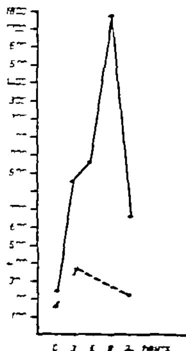


Fig. 7. Serum iron levels following intravenous injection of 500 mg. desferrioxamine and 24-hour dialysis.

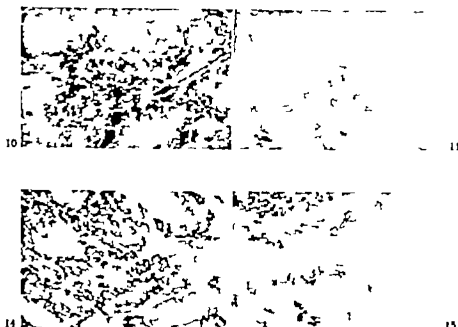


Fig. 10. Liver biopsy prior to deferrioxamine therapy (Pat. St., hereditary haemochromatosis).

Fig. 11. Liver biopsy following the removal of 18 g. iron in pat. St.

Fig. 14. Liver biopsy prior to deferrioxamine therapy in pat. F. (thalassaemia

Fig. 15. Liver biopsy following the removal of 14 g. iron in pat. F. Haemosiderin iron has largely disappeared from parenchymal and connective tissue.

Staining for iron with Turnbull blue reaction, counterstaining with haematoxylin-eosin.

When the iron-free hydroxamic acid compound, desferrioxamine B, was administered to normal test subjects, the serum iron level regularly increased in accordance with the amount injected and more iron was subsequently excreted with the urine (figs. 5a and 5b) If iron was given orally at the same time, iron excretion did not show any substantial increase, although the serum iron levels were a little higher (fig 6)

In response to desferrioxamine the urine turns salmon-pink in colour the extent of the discoloration depending on the amount of iron that has been dissolved out. This solmon pink colouring is due to dissolved ferrioxamine. Paper-chromatographic studies of urine and bile showed that the desferrioxamine-iron complex could only be demonstrated in urine. Hence, none is excreted via the bile. The unidimensional chromatograms reveal that, as was to be expected the desferrioxamine iron complex possesses the same characteristic properties as ferrioxamine.

That desferrioxamine charged with iron and circulating in the blood is in fact responsible for the rise in serum iron usually observed following administration of the substance is evidenced by the ease with which the hydroxamic acid and iron complex can be removed from the serum by dialysis the values for actual transferrin iron are then considerably lower (fig 7)

It seems particularly interesting to note that where the serum iron levels are very high, transferrin can take up iron ions from ferrioxamine until it (i. e. transferrin) is completely saturated—a finding which was confirmed by appropriate in vitro dialysis tests with pure ferrioxamine and iron-free transferrin. This result should not be considered surprising because the stability constants for desferrioxamine and transferrin differ only slightly However it would appear that iron ions can only be transferred from ferrioxamine to transferrin if the desferrioxamine is saturated to the maximum of its binding capacity On the other hand, this process easily explains why we have never observed any case of anaemia occurring in the course of desferrioxamine treatment. Apparently *therefore, despite* the mobilisation of iron there is still sufficient iron available for erythropoiesis.

From which iron-containing proteins does desferrioxamine take up iron? An exact answer to this question was obtained in dialysis tests. If pure, iron-free transferrin is introduced into a dialysis tube and ferrous sulphate into the external fluid, the ferrous sulphate

Table II

Dialysis tests with transferrin, ferritin, and haemosiderin following the addition of desferrioxamine

Substance	Concentration inside before dialysis	Concentration inside after dialysis	Iron taken up by desferrioxamine
Transferrin (human) + 20 mg desferrioxamine	225 mg. = 780.5 mcg F	34 mcg F (225 mg.)	246.5 mcg F
Ferritin (human) + 20 mg. desferrioxamine	20 mg = 4.8 mg Fe	3.63 mg Fe	1 170 mg. F
Haemosiderin (human) + 20 mg desferrioxamine	20 mg = 7 1 mg Fe	5.338 mg Fe	1 742 mg. Fe
Ferritin (horse) + 20 mg desferrioxamine	20 mg = 4.2 mg. F	2.77 mg. F	1 45 mg. Fe

Table III

Organ iron content of normal spleen and of spleen rich in iron following incubation (12 hours at 37 C.) with desferrioxamine (rabbit experiment).

	Normal spleen	Dissolved out	Iron-rich spleen	Dissolved out
	Values in mg. %			
Total organ iron	52.125		296.5	
Following addition of desferrioxamine (20 mg /200 mg. tissue)	16.325	35.8	74.5	222.0
Following addition of iron-free water	31 125	21.0	245.5	51.0

Ferritin iron, haemoglobin iron

will diffuse through the membrane and saturate the transferrin. If desferrioxamine is now added and after an incubation period of about 6 hours at room temperature, dialysis is performed against flowing distilled water the quantity of iron which is dissolved out practically attains the theoretical binding capacity of desferriox amine. Similar results were obtained with human haemosiderin and ferritin (human being and horse) This means that desferrioxamine is capable of separating and absorbing iron from all iron-bearing proteins in the organism (table II) Similarly the ability of desferrioxamine to dissolve out iron can also be demonstrated in nor mal organs and in organs rich in iron (table III)

Therapeutic Effects of Desferrioxamine

About two years ago we began to introduce desferrioxamine into therapy We would like now to give a survey of 25 treated cases,

included 13 of primary haemochromatosis, in 8 of which definite evidence of heredity was found. Our material also comprises 2 cases of thalassaemia, 1 of pulmonary haemosiderosis, 3 of cirrhoses, 1 of BLACKFAN-DIAMOND anaemia in a 4-year-old boy and 5 of achrestic anaemia with extremely severe siderosis. The results of the treatment can be seen to some extent from the iron excretion tables.

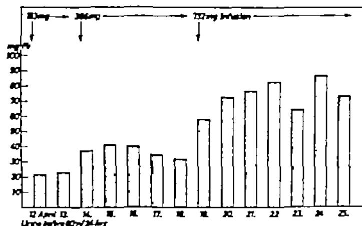


Fig. 8. Daily iron excretion following the administration of desferrioxamine (haemochromatosis).

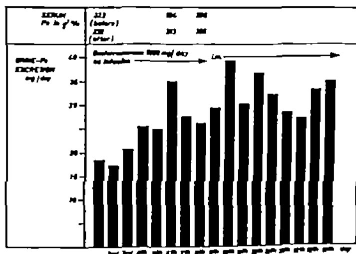


Fig. 9. Urinary iron excretion following the administration of desferrioxamine (haemochromatosis).

I one case (Pat. 5) iron excretion rose after certain initial period to 80 mg. daily in the course of the first few months, but then diminished, as figs. 8 and 9 show to levels of bout 30 mg daily I this patient pproximately 18 g iron has so far been eliminated in the space of one year

The same level of iron excretion was attained in second case of hereditary haemochromatosis over period of 14 months. Fig 10 shows the extremely severe degree of siderosis in liver biopsy material prior to the commencement of treatment. Now as fig 11 so impressively reveals, a quite considerable decrease in histochemically demonstrable liver iron has occurred The iron had largely disappeared from the cells of both parenchymal and connective tissue Similar startling improvements were achieved in other patients as well.

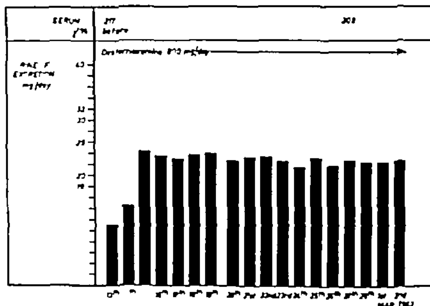


Fig 1. Urinary iron excretion following the administration of desferrioxamine in a case of haemochromatosis.

An impressive clinical feature of the treatment was that the patients often began to lose their *bronzed colour* after only 2–3 weeks. Examination of excised portions of skin in these cases tended to show a reduction in the melanin content of the basal layer rather than a definite decrease in iron. In this connection, it must be borne in mind that desferrioxamine may display a catalytic effect here since adrenaline may fail to be oxidised via adrenochrome to melanin in the presence of Fe + ions. Furthermore the rapid *subjective*

Grateful acknowledgement is made here to Prof. Caturtello who performed the liver biopsy and to Doz. Dr E. G [illegible] Chief Physician, who carried out the histochemical examination t the Institut of P thology University of Freiburg (Director Prof. F Büchner)

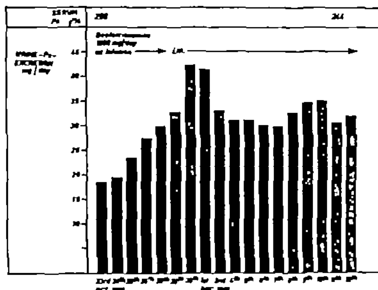


Fig 13. Urinary iron excretion following the administration of desferrioxamine, in case of thalassaemia.

improvement of the patients was most striking although in many cases (cf. table IV) the laboratory findings did not improve until some considerable time had elapsed. The very rapid subjective improvement cannot, in our opinion, be accounted for solely by the incipient excretion of iron the fact that desferrioxamine is a substance possessing oxidative properties suggests that it exerts a beneficial effect on oxidative metabolic processes.

A finding of some note in those haemochromatosis patients who had concomitant *diabetes* was that the insulin dose could be reduced within only a few days after the commencement of desferrioxamine treatment. Since, however zinc is not bound by desferrioxamine —thus excluding the possibility of the insoluble zinc-hormone insulin complex being split up—it appears conceivable that this definite reduction in the insulin requirement may be due to activation of glucose breakdown resulting from increased oxidative degradation, or possibly oxidative phosphorylation.

It was surprising to note that in some patients *iron excretion* showed, day after day almost the same levels as those recorded in patient S (fig 12)

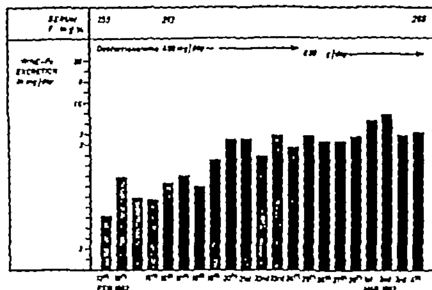


Fig. 16. Urinary iron excretion in a 4-year-old boy with BLACKFAN-DIAMOND anaemia.

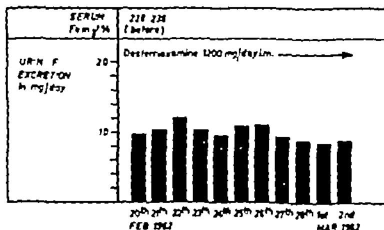


Fig. 17. Urinary iron excretion following the administration of desferrioxamine in a case of haemochromatosis.

Very good results were obtained in two cases of thalassemia, as fig. 13 shows. Altogether 14 g iron has been removed from this female patient to date. Figs. 14 and 15 illustrate the results of liver biopsy before and after treatment. As can be seen in this case too, haemosiderin iron in the liver has been tremendously reduced.

We likewise administered desferrioxamine intramuscularly to a four-year-old boy with BLACKFAN-DIAMOND anaemia who had been receiving transfusions of between 200 and 300 ml. blood every 3 weeks since his third month of life. As can be seen, iron ex-

Fig 18. Urinary iron excretion following the administration of desferrioxamine in case of liver cirrhosis.

Fig 19. Behaviour of erythrocyte and urinary porphyrins during desferrioxamine treatment in a case of sideroblastic anaemia.

cretion was very satisfactory in this case as well. Even now 7 months after the beginning of treatment, daily excretion levels of some 12 14 mg are still being recorded (fig. 16)

Understandably enough, patients who have already undergone successful venesection therapy display lower daily iron excretion levels in response to desferrioxamine (fig 17) In such cases moreover it will be possible to give smaller doses of desferrioxamine

Fig 20 Blood picture and urinary iron excretion during desferrioxamine treatment in case of pulmonary haemosiderosis.

Similarly we have observed lower iron excretion levels in cases of liver cirrhosis with siderosis of the organ. In these cases it is impossible to determine the extent to which the low iron excretion levels are due to the severe cirrhotic changes and the impaired blood flow or to the type of haemosiderin deposited (fig 18)

Of particular importance would seem to be the fact that in cases of achrestic anaemia desferrioxamine is also able to exert an effect on the *synthesis of haem.* As fig 19 impressively reveals, the pathological increase in δ-aminolaevulinic acid in urine, as well as of coproporphyrin and protoporphyrin in erythrocytes, returns practically to normal. At the same time this patient showed a rise in the haemoglobin level and erythrocyte count, reflecting an improvement in the synthesis of haem.* This process exactly paralleled the excretion of iron promoted by desferrioxamine. The account for this surprising finding, we should like to assume that the iron ions released and eliminated include some which, in the form of S-Fe-S bridges block the active SH groups of the enzymes involved in the synthesis of haem. Consequently increased enzymatic activity would be available to improve the synthesis of haem. In the case of our patient, the practical result of this process is, firstly that no more

* Common observation with Prof. Dr. Dr. L. Heilmeyer.

transfusions are required, and secondly that the severe generalised siderosis is regressing satisfactorily As may easily be imagined, the patient's general condition has shown an extraordinary improvement, he used to be unfit for work, but is now able to take up a full time occupation again.

Finally I should like to mention the iron excretion levels obtained in case of essential pulmonary haemosiderosis, in which up to 10 mg iron was eliminated daily Owing to pulmonary haemorrhages, however the treatment had to be discontinued in order to prevent an additional drain on the body's iron depot with resultant iron deficiency (fig 20).

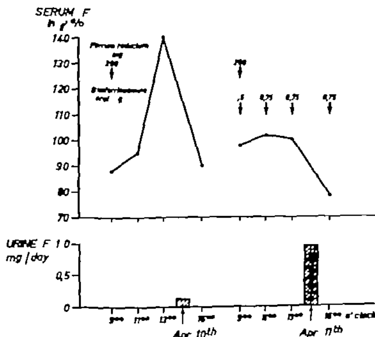


Fig 21 Serum iron levels and urinary iron excretion in response to oral administration of reduced iron with and without desferrioxamine.

Desferrioxamine medication, like venesection, still possesses one unsatisfactory feature namely that increased quantities of dietary iron are constantly absorbed during the treatment. The *increase in iron absorption* from the gastro-intestinal tract observed during treatment with desferrioxamine is due to the fact that increased amounts of iron are being dissolved out of the organism. Even

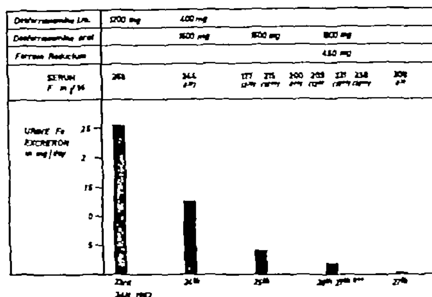


Fig 22. Absorption of reduced iron from the gastro-intestinal tract prevented by desferrioxamine in case of thalassaemia.

though this increased amount of absorbed iron were to be bound and excreted by the desferrioxamine circulating in the body an attempt should obviously be made to block iron absorption from the gastro-intestinal tract.

We therefore carried out tests with desferrioxamine B with the aim of preventing absorption of dietary iron by binding the latter in the gastro-intestinal tract. A radioactive desferrioxamine and Fe^{+++} complex was fed to rabbits by stomach tube, and it was found that this complex was not absorbed. No specific activity could be demonstrated in the serum of the experimental animals after either 4 or 12 hours.

To find out whether the absorption of iron can be blocked in the gastro-intestinal tract in human beings, we administered reduced iron to normal test subjects. The serum iron level and urinary iron excretion were measured before and after administration of the reduced iron with and without desferrioxamine, as the following figures show Whenever reduced iron was given alone the serum iron rose as expected, whereas when desferrioxamine was given simultaneously this rise did not occur (fig 21)

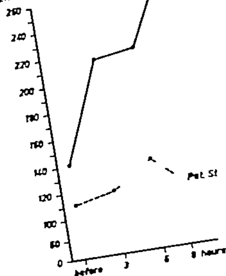
Table IV

Laboratory findings in patients St. and Sch. following 10 weeks treatment with desferrioxamine in daily dose of 800 mg.

	Pat. St. Iron excretion 6.8 g.		Pat. Sch. Iron excretion 7 g.	
	7.4.61	29.6.61	10.4.61	29.6.61
Takata	60	90	70	70
Weltmann	7½	7½	8	6
Thymol	(+)	negative	+++	negative
Total bilirubin, mg.%	2.66	1.12	1.86	0.9
Prothrombin, %	56	66	64	68
Serum iron, mcg.%	253	164	252	131
Total protein, g.%	8.5	7.6	8.0	7.9
Albumin, %	45	55	45	53
Total globulin, %	55	45	55	47
α_1-globulin, %	4	4	6	5
α_2-globulin, %	9	7	9	9
β-globulin, %	10	9	17	10
γ-globulin, %	32	25	23	23
Insulin requirement	Ø	Ø	60 U	40 U

As when desferrioxamine was administered alone, a slight excretion of iron in the urine was observed but did not exceeed 1 mg despite the massive doses of reduced iron. These tests permit the conclusion that the iron was bound in the gastro-intestinal tract and could no longer be absorbed. However a small amount of the desferrioxamine—the exact quantity cannot be determined with certainty—was doubtless absorbed and led to the increase in iron excretion as well as to the decrease in transferrin iron. Similar results were obtained in our female patient with thalassaemia who was given as much as 450 mg reduced iron by mouth (fig 22) It is certain that iron absorption was not increased. In two patients with haemochromatosis the results of administering desferrioxamine by mouth are not so easy to define (fig 23) In one case (patient Sch.) a considerable rise in serum iron was noted. Urinary iron excretion was relatively high in both cases. Perhaps it may be assumed that the amount of desferrioxamine absorbed in these cases was greater than in the other test subjects. Further absorption tests in haemochromatosis patients (figs. 24 and 25) clearly indicated, however that no iron was in fact absorbed by the gastrointestinal tract during desferrioxamine medication, while in controls an increase of the serum iron levels was observed.

This is evidenced in particular by the fact that following the administration of radioactive iron serum radioactivity was almost



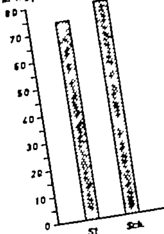


Fig 23. Serum iron levels and urinary iron excretion following oral administration of 3 capsules (= 750 mg) desferrioxamine

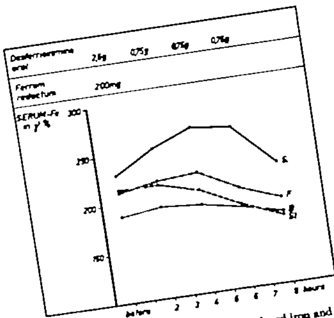


Fig 24 Serum iron levels following administration of reduced iron and desferrioxamine in haemochromatosis patients.

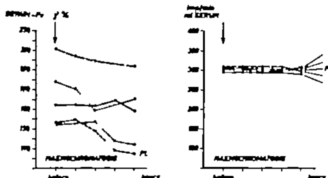


Fig. 25. Serum iron levels and radioactivity in the serum following oral administration of 20 mg. Fe^{++} ions (20 μc. Fe^{59}) and of deferrioxamine (2 g.) in cases of haemochromatosis.

always nil and no increase could be noted. Somewhat similar findings are recorded when absorption tests are carried out by administering reduced iron or ferrous sulphate in the form of biscuits containing 200 mg iron ions.

To sum up the combined parenteral and oral administration of desferrioxamine makes it possible both to remove iron from the human organism and to prevent iron being absorbed. This appears to be an ideal combination for the future treatment of haemochromatosis.

Summary

A new method of treating iron storage diseases is reported. The method is based on the principle that iron ions in the organism can be specifically bound by desferrioxamine, hydroxamine acid compound, and then, owing to the low molecular weight of the resultant complex, eliminated via the kidney. Other trace elements are not affected by desferrioxamine. Experimental data are given on iron excretion in animals, as well as on the extent to which desferrioxamine dissolves out iron from transferrin, ferritin, and haemosiderin. In human beings, iron excretion levels of up to 80 mg. daily have been recorded, with an average of bout 30 mg. daily. The patients treated comprised cases of primary and secondary haemochromatosis, achrestic anaemia, liver cirrhosis, Blackfan-Diamond anaemia, and pulmonary haemosiderosis. Since desferrioxamine also inhibits enteral absorption of iron, the ideal treatment would seem to consist in administering the drug both intramuscularly (500 mg. 2 times daily) and orally in the form of capsules to be taken with meals (250 mg. 3 times daily).

Résumé

Description d'une nouvelle méthode de traitement des thésauroses du fer. La méthode se base sur le principe que les ions du fer sont spécifiquement fixés dans le corps par la desferrioxamine, un composé de l'acide hydroxamique, et que le complexe for

mé, à cause de son poids moléculaire peu élevé, est éliminé par les reins. D autres oligo-éléments ne sont pas influencés par la desferrioxamine. L auteur rapporte des résultats des expériences sur l'excrétion d fer par des animaux dont découle, que la desferriox amine dissout le fer dans la transferrine, la ferritine et l'hémosidérine. Chez l'homme, une excrétion journalière jusqu'à 80 mg pu être observée, la moyenne journalière est de 30 mg. Les malades traités souffraient d'hémochromatose primaire ou secondaire, d'anémie achrestique, de cirrhose hépatique, d'anémie du type Blackfan-Diamond et d'hémosidérose pulmonaire. Parceque la desferrioxamine entrave l' bsorption intestinale du fer le traitement idéal semble être l'administration intramusculaire (400 mg 3 fois par jour) et orale sous forme de capsules pendant les repas (250 mg 3 fois par jour).

Zusammenfassung

Es wird über eine neue Methode zur Behandlung der Eisenspeicherkrankheit berichtet, die darauf beruht, daß durch Desferrioxamin, eine Trihydroxamsäureverbindung, Eisenionen im Organismus spezifisch gebunden werden können und infolge des kleinen Moleküls durch die Niere entfernt werden. Andere Spurenelemente werden nicht erfaßt. Experimentelle Ergebnisse über die Eisenausscheidung beim Tier die Herauslösung von Eisenionen aus Transferrin, Ferritin und Hämosiderin werden mitgeteilt. Die Ergebnisse beim Menschen zeigen Eisenausscheidungen bis zu 80 mg maximal, im Durchschnitt etwa 30 mg täglich. Behandelt wurden primäre und sekundäre Hämochromatosen, siderochrestische Anämien, Leberzirrhosen, eine Blackfan-Diamond-Anämie sowie eine Lungenhämosiderose. Da das Desferrioxamin auch die enterale Eisenresorption hemmt, erscheint als ideale Therapie einmal die intramuskuläre Verabreichung (zweimal 500 mg pro die i. m.) und die orale (dreimal 250 mg pro die) als Kapsel zu den Mahlzeiten.

Literatur

1 Bickel, H. Gäumann, E. Keller-Schierlein, W Prelog, V Vischer, E.; Wettstein, A. und Zähner, H. Über eisenhaltige Wachstumsfaktoren der Sideramine und ihre Antagonisten, die eisenhaltigen Antibiotika Sideromycine. Experientia *16* 129 (1960)

2. Bickel, H. Hall, G. E. Keller-Schierlein W ; Prelog, V ; Vischer, E. und Wettstein, A. Über die Konstitution von Ferrioxamin B. Helv chim. Acta *18*. 261 (1960)

3. Clatton F J Noyes, W D ; Turnbull, A. and Finch, C. A. The use of chelates in the treatment of iron overload. Clin. Res. *9* 91 (1961).

4 Fahey J Princiotto, J Rath, C. and Rubin, M. Evaluation of new chelate (DTPA) in iron storage disease. Clin. Res. *8* 52 (1960)

5. Figueroa, W G and Tuttle, S. G. A study of iron excretion following Ca DTPA administration. Clin. Res. *9* 92 (1961)

6. Foreman, H. The pharmacology of some useful chelating agents In Seven's and Johnson' Metal-Binding in Medicine pp. 82–94 (Lippincott, Philadelphia 1960).

7 Gerhold, E. und Puxkandl, H. Die Beeinflussung der renalen Eisen- und Kupferausscheidung. Wien. Z. inn. Med. *36*. 491 (1955).

8. Palmieri, A. und Giacca, S. Neuorientierung der Behandlung des Haemochromatose-Syndroms. Schilderung eines Falles, der mit Dioxyäthylglycin behandelt wurde. Arch. E. Maragliano Pat. *11* 993–1011 (1955)

9 Rubin, M. and Princiotto, J V. Synthetic aminoacid chelating agents and iron metabolism. Ann. N Y Acad. Sci. *88*. 450–459 (1960)

10. Vischer, E. Chemie des Desferrioxamin B. Meeting of clinical investigators held at CIBA, Basle 1961

11. Wöhler, F. Klinisch-experimentelle Ergebnisse mit Desferrioxamin. Meeting of clinical investigators held at CIBA, Basle 1961

12. Wöhler, F. Über die Freisetzung von Eisen aus dem menschlichen Organismus durch eine Hydroxamsäureverbindung. Proc. 8th. Congr. europ. Soc. Haemat., p. 244 (Karger, Basle/New York 1961).

13. Wöhler, F.: Therapie der Haemochromatose. 8. Deutsche Hämatologen-Tagung, Wiesbaden 1962.

14. Wöhler, F.: Die Therapie der Hämochromatose. Med. Klinik 57: 1370 (1962).

Author's address: Doz. Dr. F. Wöhler, Dept. of Medicine, Hugstetterstr. 55, *Freiburg i. Br.* (Germany).

Acta haemat. 30: 88–102 (1963)

From the Medical Clinic of the University of Perugia
(Director: Prof. P. Larizza)

Congenital Non Spherocytic Haemolytic Anaemia Due to Pyruvate-Kinase Deficiency

By Paolo Brunetti, Adolfo Puxeddu, Giuseppe Nenci and Ermanno Migliorini

Congenital non spherocytic haemolytic anaemias represent a heterogeneous group of diseases with certain common fundamental features, such as lack of spherocytosis, normal haemoglobin, normal osmotic fragility, little if any benefit by splenectomy and apparent unsteadiness of family distribution. Remarkable progress was made in the last decade in the study of etiology of this haemolytic diseases through metabolic and enzymatic investigations. Since 1953 Dacie et al. (6) emphasized the presence of increased autohaemolysis in vitro and of some anomalies of erythrocyte morphology. In 1954 Selwyn and Dacie (33) distinguished two types of congenital non spherocytic haemolytic anaemias (type I and type II) upon the basis of the different behaviour of erythrocytes during incubation in vitro. In type I autohaemolysis and loss of potassium were normal or only slightly increased and the corrective action of glucose was evident but usually less than in normal erythrocytes. Type II erythrocytes were instead characterized by a markedly increased autohaemolysis and by a heavy potassium depletion after incubation. On the other hand addition of glucose did not correct any of these alterations.

This research was supported by a contribution of the Italian National Council of Research.

The following abbreviations have been used in the text: ADP = adenosin-diphosphate, AMP = adenosin-monophosphate, ATP = adenosin-triphosphate, 2,3-DPG = 2,3-diphosphoglycerate, DPN = diphosphopyridinnucleotide, F-6-Pk = fructose-6-P kinase, GAPD = glycerinaldehyde-3-P-dehydrogenase, GPD = glycerophosphate-dehydrogenase, G-6-PD = glucose-6-P-dehydrogenase, GSH = reduced glutathion, GSSG = oxidized glutathion, LD = lactic-dehydrogenase, MD = malic dehydrogenase, 6-P-GD = 6-phosphogluconate-dehydrogenase, PGK = phosphoglycerate-kinase, Pk = pyruvate-kinase, TPN = triphosphopyridinnucleotide.

Some observations revealed a defective glucose metabolism in type II erythrocytes. SELWYN AND DACIE (33) found an abnormally low utilization of glucose, and DE GRUCHY et al. (8 9) demonstrated a decrease of the ATP content. It was subsequently found that spontaneous autohaemolysis in vitro in type II cases, not correctible by glucose, could be normalized by addition of ATP to incubated blood (9) In patients with congenital non spherocytic haemolytic anaemia a defective incorporation of P^{32} into ATP has been demonstrated by PRANKERD (26) This was confirmed by ROBINSON et al. (31) who observed an accumulation of 2,3-DPG in type II erythrocytes and assumed an enzymatic block within the glycolytic chain following 2,3-DPG synthesis.

In patients affected by this haemolytic anaemia, VALENTINE et al. (36) found for the first time a lack of pyruvate kinase (PK) in erythrocytes and showed the hereditary character of this deficiency (34) BRUNETTI et al. (3) revealed the results of an enzymatic and genetic study performed on one of their patients with congenital non spherocytic haemolytic anaemia due to PK deficiency Similar observations were subsequently reported by PRANKERD (28) and by LÖHR AND WALLER (22)

The present paper reports the results of a study performed on two patients with congenital non spherocytic haemolytic anaemia. In both a hereditary erythrocyte PK deficiency was demonstrated.

Materials and Methods

Routine haematologic studies were performed by standard methods. Autohaemolysis was studied according to SELWYN AND DACIE (33) with the detailed procedure reported by BRUNETTI et al. (3). The various enzymatic activities, PK included, were determined in the haemolysat according t the methods described by BRUNETTI et al. (3). The method of BÜCHER AND PFLEIDERER (4) for PK assay was modified by the addition of GSH, by which higher and more easily reproducible values are generally obtained. GSH, total pentoses and aldopentoses were determined according to the methods of GRUNERT AND PHILLIPS (12), MEJBAUM (23) and DISCHE AND BORENFREUND (10) respectively Determination of sialic acid (N-acetyl-neuraminic acid) was based on the method of HESS et al. (14) as described by BRUNETTI AND PUXEDDU (2). Stroma preparations were performed according to the method of TUCHKOFF et al. (35) for sialic acid determinations and of DANON et al. (7) for activation experiments.

Case Histories

Case I G. Fernando, 43 years old man, presented the first episode of jaundice during his first year of life an icteric colouring in sclerae had always been evident, though subject to mild variations in intensity At 22, while performing his military service, splenomegaly was diagnosed for the first time. Ever since his 35th year of age

Table I
Haematological data in patients with pyruvate kinase (PK) deficiency

	Erythrocytes (range) mill./mm³	Haemoglobin (range) g /100 ml.	MCV μ^3	MCHC %
Case 1				
Before splenectomy	2.60–3.47	8.00–11.04	134	24
After splenectomy	3.00–3.41	10.00–11 10	135	26
Case 2	2.81 3.79	11 14–12.16	110	33

he had shown progressive asthenia and fatigue. These symptoms accentuated in the first months of 1962 and at the same time, such an increase in the icterus was observed to lead him to first admission into hospital, where haemolytic anaemia with splenomegaly was diagnosed (spleen palpable 4 cm. below the left costal margin) RBC ranged between 3 and 3.5 millions and serum bilirubin was not higher than 4 mg.% On that occasion, splenectomy was suggested to the patient. H was brought to our attention during hospitalization in the surgery department. During and after surgery one liter of blood was transfused. Morphological and enzymatic studies were performed both before and after operation. The spleen weighed 550 g after squeezing of the blood. Splenectomy did not yield essential improvement in the patient state. Haemolysis was just about unchanged as shown by the persistently low values of the erythrocyte counts, high reticulocytosis and by the oscillations of bilirubinaemia. The most significant haematologic data obtained during hospitalization are shown in table I. Neither the patient's parents nor his relatives, including his children, ever presented icteric episodes relating to haemolytic anaemia.

Case 2. L. G. Marina, a 16-year-old girl, was admitted to our Clinic on 15th November 1962. According to her parents, mild pallor of the skin and mucous membranes had always been evident. Icterus was first found in August 1962. Relevant asthenia and fatigue were noted contemporaneously Urine got darker while her skin pallor accentuated. A mild splenomegaly (inferior pole palpable slightly below the thoracic arch) and moderate liver enlargement were present. Remarkable oscillations in icterus intensity were noted, ever since it was first revealed. Blood transfusions up to 1 litre were performed during hospitalization. The most significant haematologic data obtained t the time of hospitalization are summarized in table I. No other member of the family including her parents and 2 year-old sister had ever presented episodes of icterus.

Table II
Autohaemolysis studies in patients suffering from pyruvate-kinase (PK) deficiency haemolytic anaemia. Results are expressed in % haemolysis after 48 hours incubation at 37 °C.

Added compound	None	Glucose	ATP	ADP	AMP	Adenosine
Case 1	23.00	11.00	1.00	2.82	2.25	1.30
Case 2	5.80	4.30	1.80	2.20		4.50
Normal average	3.54	0.56	0.49	0.38	0.28	0.33

Table I (continued)

MCH	MCD	Reticulo-cytes	Bilirubin	Osmotic fragility % NaCl	
μμg	μ	(range) %	(range) mg. %	before incubation	after incubation
30	7.76	5–6	1.30–3.80	0.30–0.44	0.24–0.58
33	7.78	6–10	0.60–2.70		
37	7.51	4–8	3.70–4.75	0.32–0.44	0.26–0.64

Results

A summary of the most significant haematologic data is shown in table I Both patients revealed mild to moderate normochromic anaemia with moderate hyperbilirubinemia and reticulocytosis. In both cases irregularly edged erythrocytes were found some of which were hyperpigmented and irregularly contracted while others were of a triangular shape, together with a prevalent macrocytosis and moderate polychromasia. In case 1 there were no significant changes after splenectomy except for an increase of the leptocytosis, present before the operation and a remarkable number of siderocytes (81 %) In case 2 a small number of elliptocytes was also observed. In both cases, the osmotic fragility of fresh blood was normal. After incubation at 37 C. for 24 hours it was slightly decreased.

Table III

Pyruvate-kinase (PK) activity of erythrocytes from patients with congenital non spherocytic haemolytic anaemia (homozygotes) from their relatives believed to be heterozygotes and from normal persons. PK values are expressed as units defined as μMoles of phosphoenolpyruvate used in one minute by 10^{11} RBC.

	Number studied	Mean and S.D	Range
Patients *Case 1*		4.17	
Case 2		4.36	
Heterozygotes	25	10.4 ± 2.87	6.8–15.6
Normals	70	19.61 ± 4.19	13.4–31.3

The results of autohaemolysis studies are shown in table II An increase of autohaemolysis in respect to normal was observed in both cases after 48 hours. It was only partially corrected by glucose

Table II

ATP ADP and AMP content of erythrocytes from patients with pyruvate-kinase (PK) deficiency subjects believed to be heterozygotes and normal persons. Values are expressed in µMoles per 100 ml. RBC.

	Number studied	ATP	ADP	AMP
Patients				
Case 1		80.9	70.5	17 7
Case 2		94 0	42.7	23.2
Heterozygotes (Kindred 2)				
Mean and S. D	7	126 ± 11.83	17 4	14.5
Range		113–146	7.5–34.5	12.0–17.2
Normals				
Mean and S.D	10	146.5 ± 3.71	41 4	19.2
Range		134–168	32.9–68.2	7 4–28.1

but was brought back to normal by ATP To be noted are the positive results obtained in both cases with ADP and AMP and in case 1 only the efficacy of adenosine.

PK determinations were performed on 70 normal individuals (males and females) ranging in age from 15 to 70 years. Mean values, extremes of variations and standard deviation are shown in table III It is evident that both patients had a very low PK content in erythrocytes. Preliminary experiments do not provide evidence for the presence of an inhibitor or the lack of an activator in the pathologic haemolysate Mixture of normal haemolysates with the haemolysates of patients with PK deficiency yielded a PK activity equal to the sum of the activities of the separate specimens. The same results were obtained both before and after incubation of the mixture at 37 C, for periods ranging from 15 to 60 minutes. Similarly crystalline PK activity was not modified by incubation with pathologic haemolysate. Finally stromata obtained from normal individuals did not prove to be activators of the pathologic haemolysate

Together with the marked decrease of PK activity a decrease of the erythrocyte ATP content up to 40 / of the normal mean was also noticed. No changes in the erythrocyte content of ADP and AMP were observed (table IV)

All the other enzymes of oxidative and anaerobic glycolysis, besides PK, did not differ significantly from the average mean

Table V

Summary of the activities of glycolytic enzymes investigated in the erythrocytes of patients with congenital non-spherocytic haemolytic anaemia. Enzyme values are expressed in units defined as μMoles of substrate used in one minute by 10^{11} RBC.

Enzymes	*Case 1*	*Case 2*	Normal	
			Mean	Range
PK	4.1	4.5	19.61	13.4–31.5
G-6-PD	12.9	14.9	19.30	13.6–26.7
6-P-GD	5.1	—	5.26	4.7–6.0
F-6-PK	15.4	20.2	20.1	16.9–27.8
Aldolase	6.5	6.1	5.9	4.9–6.5
GAPD	189.0	188.0	264.0	191.0–370.0
PGK	480.0	403.0	590.0	505.0–835.0
GPD	1.5	0	0	
Enolase	35.4	15.2	31.7	25.8–39.5
LD	*261.0*	*528.0*	*233.0*	*210.0–274.0*
MD	214.0	246.0	232.0	220.0–252.0

Table VI

Total pentoses, aldopentoses and sialic acid (N-acetyl-neuraminic acid) content of erythrocytes from patients with pyruvate-kinase (PK) deficiency. Pentoses are expressed in μg per ml. RBC and sialic acid in μg. per mg of stromata.

Subjects	Total pentoses	Aldopentoses	Sialic acid
Case 1	136.0	91.0	9.44
Case 2	220.0	137.6	11.41
Normal mean	344.5	221.7	11.42
Range	246–431	193–283	10.84–13.26

(table V) The erythrocyte GSH and its stability after incubation with acetylphenylhydrazine were normal.

A marked reduction of the total pentoses and aldopentoses content of PK-deficient erythrocytes was also observed of the same order as the ATP diminution (table VI). A slight decrease of sialic acid in stromata was shown in case 1 only (table VI).

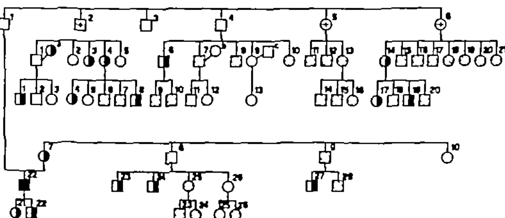
Studies performed on our two patients' blood-relatives demonstrated that the PK defect has a family and hereditary character. PK activity was determined in 42 members of the two family groups. The individuals observed can be divided into two groups according to whether PK values can be placed above the normal area (18.4 u., on an average) or whether they are below it (10.4 u., on an average) (tables III, VII). Separation between the two groups on the basis of

Table VII

Erythrocytic PK activity in the relatives of patients. Subjects are grouped in two classes with normal and intermediate PK values. Symbols refer to the pedigrees (fig. 1). PK values are expressed as μMoles of phosphoenolpyruvate used in one minute by 10^{11} RBC.

Normal subjects	PK values	Hetero-zygotes	PK values
Family of Case 1			
II b	17.3	I 7	13.2
II	20.0	II	12.7
II 1	20.5	II 3	11.2
II 2	20.4	II 5	11.2
II 9	16.5	II 6	10.8
II 26	16.4	II 14	12.0
III 2	22.0	II 23	10.50
III 3	21.2	II 24	12.4
III 5	19.2	II 27	9.0
III 11	20.8	III 1	13.6
III 12	20.5	III 4	10.8
III 13	21.2	III 8	8.8
III 18	16.2	III 17	10.6
III 25	16.1	III 19	12.4
		III 21	9.9
		III 22	8.0
Family of Case 2			
II 4	15.8	II 1	12.1
III 3	15.6	II 2	12.4
III 4	15.2	II 5	11.2
		II 6	9.3
		II 7	7.9
		II 8	6.9
		II 9	10.9
		III 10	9.2
		III 14	13.3
Mean	18.4		10.8
Normal mean and range 19.6 (13.4–31.5)			

PK activity is not clear since there exists an overlapping zone between 13 and 14 units. Subjects with intermediate PK activity must be considered as probably being heterozygous for the pathologic gene whereas to patients with haemolytic anaemia and marked lack of PK a condition of homozygosity for the same can be ascribed. Evidence of hereditary transmission of a semi-dominant autosomic type was represented by the demonstration of an inter



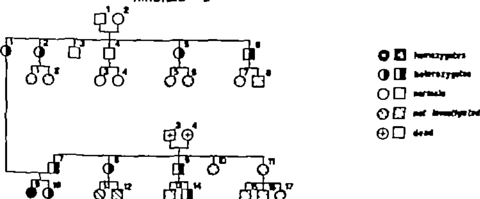


Fig 1. Family trees of the two patients affected by PK-deficiency haemolytic anaemia. Males are represented with squares and females with circles.

mediate PK deficiency in the mother and in both the children of case 1 and in both the parents of case 2 (fig 1)

It is to be noted that none of the individuals with intermediate PK values revealed, at the time of examination or in the past, any clinical evidence of anaemia or of hyperhaemolysis. Nevertheless, ATP content in 7 heterozygous subjects, family members of case 2, was 126 μM per 100 ml. of packed RBC, that is below the normal mean of 146.5 μM. The difference between the two values appears significant with 2p between 0.001 and 0.005 If these findings should

be confirmed a three-group-classification would be made The first would comprise homozygotes for the pathologic gene with low if any PK activity and remarkable ATP decrease the second consistent of heterozygotes with intermediate PK and ATP values and the third with normal values. In intermediates no change in the other glycolytic erythrocyte enzymes was observed

Discussion

Until now more than 80 cases of congenital non spherocytic haemolytic anaemia have been described On the basis of autohaemolysis tests only a minority of these can be considered as belonging to type II In a review of the literature until 1962 BRUNETTI et al. (3) listed 17 of these cases (including case 1 of the present report) in which the diagnosis was made with a sufficient degree of certainty (6 9 15 34 20, 25) and others in which the connection with type II could be considered probable but not proved (1 24 32 13) Among these cases of congenital non spherocytic haemolytic anaemia of type II a lack of PK was observed in 7 patients by TANAKA et al (34) in the two patients who are the subject of the present study and more recently in other cases by PRANKERD (28) and LÖHR AND WALLER (22)

It is likely that other enzymatic defects, besides that of PK, account for the particular behaviour of autohaemolysis in type II cases, but undoubtedly there exists a remarkable uniformity of clinical and haematological features within the same group A greater occurrence of congenital non spherocytic haemolytic anaemia in persons of Northern-European stock has been admitted for a long time in fact with the exception of the Mexican patient studied by TANAKA et al (34) and of our own two patients who belong to Umbrian families, all subjects with PK deficiency are of Northern origin. The lack of a systematic study of the distribution of the genetic defect in either its homozygous or heterozygous form, however impedes us from attributing a typically racial character to this new enzymatic defect

The first symptoms of haemolytic disease in subjects with PK deficiency appear in most cases, in the period immediately after birth, as in early infancy much more rarely at a more advanced age and never after 20 years of age The seriousness of the haemolytic form is extremely variable the most frequent cases being those with

a notable degree of anaemia and with a great need for transfusions. DE GRUCHY et al. (9) underlined a difference in the clinical course of congenital non spherocytic haemolytic anaemia, in type I and type II cases, attributing a greater precocity of evolution and more accentuated haemolytic phenomenology to the latter Some of the cases described by TANAKA et al. (34) and the two patients described by us, displayed on the contrary a moderately mild course and an extremely small transfusional need.

The observations of the various authors are sufficiently in agreement in attributing a character of moderate macrocytosis to type II congenital non spherocytic haemolytic anaemia. DE GRUCHY et al. (9) and DACIE et al. (6) called attention to the presence of crenated or irregularly contracted erythrocytes. Cells with these features were present in both cases studied by us. Evident morphological anomalies were not encountered however by TANAKA et al. (34) Evaluation of osmotic fragility generally normal or decreased in both fresh and incubated blood, helps in the diagnostic definition of these haemolytic forms, since it permits to differentiate the latter from manifest or latent cases of hereditary spherocytosis.

Greater importance must be given to the simple test of spontaneous haemolysis of blood incubated in vitro at 37 C. for 24 to 48 hours, with or without the addition of glucose or ATP In haemolytic anaemia due to PK deficiency identifiable with type II congenital non spherocytic haemolytic anaemia, autohaemolysis is notably increased and glucose is capable only of a slight, or of no, corrective capacity ATP on the contrary is capable of preventing completely the haemolysis (9) TANAKA et al. (34) demonstrated that ADP and AMP are also capable of an analogous protective power shared, at least partially by other compounds such as DPN TPN GSH and CoA. It was, moreover noticed that adenosine can also partially correct spontaneous hyperhaemolysis.

Increase in autohaemolysis and the correction performed by ATP are directly correlated to the original enzymatic defect. In fact the only gain in energy of anaerobic glycolysis is obtained on a level with the PK reaction, since the production of ATP deriving from the GAPD reaction compensates the ATP loss linked to the phosphorylization of glucose and 6-P fructose. The phosphorylated compounds of adenosine would be effective in preventing hyperhaemolysis of PK deficient erythrocytes in vitro, because, through a pathway different from that which is habitual in anaerobic glyco-

lysis they lead to the reintegration of the erythrocyte ATP supply. The added ATP would first undergo dephosphorylization to ADP by the action of a phosphatase of the plasma, and then be converted to an equi-molecular quantity of AMP and ATP in the erythrocyte membrane by an adenilate kinase present there (16).

The defect of the ATP synthesis seems up to now the most severe metabolic consequence due to PK deficiency. Since it is from ATP that erythrocytes derive their energy for the most important vital functions (maintenance of a cationic gradient etc.) it appears logical that lack of it could assume an essential role in the pathogenesis of haemolysis. A study of splenic histology in PK deficiency haemolytic anaemia has demonstrated absence of any signs of erythrostasis in the sinuses which is, on the contrary, a constant feature of hereditary spherocytosis (3). In case 1, where a histological study of the spleen showed the above picture, splenectomy did not bring about any change in hyperhaemolysis. From this it appears that haemolysis presents a fundamentally intra vascular feature in PK deficiency haemolytic anaemia. It is still unknown if other factors, besides the mentioned decrease in ATP synthesis, can participate in the determination of haemolysis, or if the severity of the haemolytic anaemia in different patients can be correlated with the degree of the enzyme defect. Although Tanaka et al. (34) noticed a remarkable fluctuation in PK values among patients with haemolytic anaemia, they believe that the intensity of haemolysis can be more easily correlated with the simple auto-haemolysis tests, rather than with the enzyme activity. However, it seems essential for the production of haemolysis that the PK activity values remain below those of aldolase and hexokinase which, being the lowest in the glycolytic chain act as pacemakers in the total speed of the metabolic cycle. It seems also likely that all the factors capable of determining an alteration of the activity ratio between PK and hexokinase can modify the degree of hyperhaemolysis in subjects with the enzyme defect.

The experimental data so far acquired lead to ascribe the decrease in PK activity to an actual enzyme defect. Activation studies performed by Tanaka et al. (34) and by us, employing haemolysates and normal stroma preparations, with or without addition of SH-compounds, like GSH or cysteine gave no evidence for the presence of inhibitors or the absence of physiologicals activators of PK activity in erythrocytes with PK deficiency. On the

contrary as far as the erythrocyte G-6-PD deficiency is concerned, the absence of a factor activating the enzyme in the stromata of enzyme-deficient erythrocytes has been suggested by several workers (30 29 11)

Determinations of PK performed in leukocytes (34) and in bioptic fragments of hepatic and muscular tissue (3) in patients with PK deficiency haemolytic anaemia, did not reveal an extension of the enzyme defect to cellular elements other than erythrocytes. This is not surprising since erythrocytes, by reason of their structure and of the poverty of their metabolic processes, display in respect to nucleated cells with a more active metabolism, a much greater predisposition for specific enzyme-defects induced by genetic aberrations. The G-6-PD-deficiency in subjects of white race reveals itself complete in erythrocytes but only partial in liver tissue (3)

In family members of patients with haemolytic anaemia the PK deficiency is milder than in the patients themselves. These intermediate values of PK activity are related to a heterozygous condition for the pathological gene. The remarkable difference among intermediate subjects leads to assume a great variability in penetration and expressiveness of the pathological gene in the heterozygous condition, as it has been repeatedly observed in G-6-PD deficiency (18, 19) On this basis, the family trees, in which the enzyme defect seems to be transmitted as a semi-dominant autosomal gene, can be easily interpreted. From the data collected by Tanaka et al. (34) and from our own experience as well, it can be concluded 1 If both parents are intermediate (that is heterozygote for the pathological gene) they can have children who are also intermediate or normal, or affected by the PK deficiency in its severest form, i.e. the haemolytic disease. 2 If one of the parents is normal and the other a homozygous carrier of the defect, all children will be heterozygotes. 3 If only one of the parents is intermediate, the children can be either normal or intermediate. The reverse condition is also true, since the enzymatic defect has never been encountered with, in total or partial form, without being parallely documented also in the ancestors of the subjects in question.

PK-deficiency haemolytic anaemia is logically included in the group of enzyme-deficiency haemolytic anaemias. This term, originally applied to G-6-PD deficiency haemolytic forms (17 18) must now be extended to the anaemias of more recent *individualiz*

ation due to lack of GSSG-reductase (5 21) of diphosphoglycero-mutase (27 37) and of PK. It is also very likely that the group of enzyme-deficiency haemolytic anaemias will be enlarged by the extension of enzymatic studies to other haemolytic forms which have so far been of difficult classification.

Summary

In two patients affected by congenital non spherocytic haemolytic anaemia of type II a deficiency of pyruvate-kinase (PK) has been demonstrated. PK-deficient erythrocytes show also a marked decrease of their content in ATP total pentoses and aldopentoses. Other glycolytic enzymes, as well as glutathion, appear to be normal. The PK-deficiency seems to be transmitted through a semi-dominant autosomic gene. Heterozygous subjects, within the families of the two patients, are characterized by intermediate values of PK.

Résumé

L existence d'un manque de la pyruvate-kinase (PK) pouvait être démontré bez deux malades, teints d'une anémie hémolytique non sphérocytaire congénitale du type II. A part ce défaut de la PK les érythrocytes étaient caractérisés par une diminution de l'ATP des pentoses totales et des aldo-pentoses. L activité des utres ferments glycolytiques et la concentration d glutathion paraissent être normales. Le manque de la PK est transmis par un gène autosomal sémidominant. Des parents hétérozygots des malades présenten des valeurs intermédiaires.

Zusammenfassung

Bei zwei Pa ienten mit kongenitaler nicht sphärocytärer hämolytischer Anämie Typ II wurde ein Mangel an Pyruva kinase (PK) nachgewiesen. Neben dem PK Mangel zeigen die Erythrozyten eine deutliche Verminderung des ATP der Gesamt Pentosen und der Aldopentosen. Die Aktivität der anderen glykolytischen Enzymen und der Glutathion-Gehalt liegen im Bereich der Norm. Der PK Mangel wird durch ein autosomales semidominantes Gen übertragen. Heterozygot Verwandt der Patienten zeigen in ermediäre PK Werte

References

1 Baty J M. A case of congenital hemolytic jaundice wi h an unusually high percentage of reticulocytes. Amer J med. Sci. 179 546 (1930)

2. B unetti, P Peirnoo A. Prime ricerche sul comportamento dell'acido stalico eritrocitario nella etalassemia major nel favismo ittero-emoglobinurico. Minerva med. 53 1627 (1962)

3. Brunetti, P Peirnou A. e Nenci, G. Anemia emolitica congenita non sferocitica da carenza di piruvico-chinasi (PK) Haematologica 47 505 (1962)

4 Bücher, T and Pfleiderer, A. Pyruvat kinase from muscle In Colowick's and Kapla Methods in Enzymology vol. 1 p. 435 (Academic Press, New York 1955).

5. Carson, P E.; Brewer, G J and Ickes, C. Decreased glutathione reductase with susceptibility to hemolysis. Cent. Soc. clin. Res. 19 995 (1961)
6. Dacie, J V; Mollison P L.; Richardson, N Selwyn, J G and Shapiro, L. Atypical congenital haemolytic anaemia. Quart. J Med. 22: 79 (1953)
7 Danon D.; Nevo, A. and Marikovsky Y Preparation of erythrocyte ghosts by gradual haemolysis in hypotonic aqueous solution. Bull. Res. Counc. Israel 6: 36 (1956).
8. De Gruchy G. C.; Crawford, M. and Morton D Atypical non spherocytic haemolytic anaemia. Paper 198. 7 Congr int. Soc. Haemat., Rome 1958.
9 De Gruchy G C.; Santamaria, J N; Parsons, I. C. and Crawford, H. Non-spherocytic congenital hemolytic anaemia. Blood 16: 1371 (1960)
10. Dische, Z. and Borenfreund, E. A new colour reaction for the determination of aldopentose in presence of other saccharides. Biochim. biophys. Acta 23. 639 (1957).
11 Grignani, F und Brunetti, P Weitere Untersuchungen über die Eigenschaften des aktivierenden Faktors der Glucose-6-Phosphat-Dehydrogenase der menschlichen roten Blutkörperchen bei hämolytischen enzymopenischen Anämien. Folia haemat. N. F 7 24 (1963)
12. Grunert R. R. and Phillips, P H. A modification of the nitroprusside method of analysis for glutathione. Arch. Biochem. 30: 217 (1951).
13. Hiddemann, H. H. Atypische kongenitale haemolytische Anämie. Z. ges. inn. Med. 10 633 (1955)
14. Hess, E. L. Coburn, A. F; Bates, R. C. and Murphy P A new method for measuring sialic acid levels in serum and its applications to rheumatic fever J clin. Invest. 36. 449 (1957)
15. Horswall, W R. A case of congenital non-spherocytic haemolytic anaemia presenting with an plastic crisis. Med. J Austr 11 340 (1956).
16. Kashket S. and Denstedt O. F The metabolism of the erythrocyte. XV Adenylate kinase of the erythrocyte. Canad. J Biochem. Physiol. 36. 1057 (1958).
17 Larizza, P Hämolytische enzymopenische Anämien. 7 Kongr dtsch. Ges. Hämat., Wiesbaden 1961.
18. Larizza, P; Brunetti, P Grignani, F Anemie Emolitiche Enzimopeniche. Tipografia Viscontea, Pavia 1959.
19. Larizza, P; Brunetti, P Grignani, F Ventura, S. L'individualità bio-enzimatica dell'eritrocito «fabico». Haematologica 43 205 (1958).
20. Lipow J L.; Sachar, J H. and Daniels, J H. Congenital nonspherocytic hemolytic anemia. Report of two cases. Amer J clin. Path. 37 606 (1962)
21. Löhr, G W und Waller, H. D. Eine neue enzymopenische hämolytische Anämie mit Glutathionreduktase-Mangel. Med. Klin. 57 1521 (1962).
22. Löhr, G. W and Waller, H. D. Pers. commun.
23. Mejbaum, W Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. Z. physiol. Chem. 258. 117 (1939)
24. Nelson, M. G. Atypical congenital hemolytic anemia. Arch. Dis. Child. 29: 457 (1954).
25. Nordöy A.; Brewer, J; Myhre, E. and Solberg, S. Studies on glycolysis in hereditary non-spherocytic hemolytic disease. Acta med. scand. 172: 67 (1962)
26. Prankerd T A. J Inborn errors of metabolism in red cells of congenital hemolytic anaemias. Amer J Med. 22: 724 (1957).
27 Prankerd, T A. J. The Red Cell. An Account of its Chemical Physiology and Pathology Blackwell Scient. Publ., Oxford 1961.
28. Prankerd, T A. J Pers. commun.

29. Ramot, B.; Ashkenazi, J.; Rimon, A.; Adam, A. and Sheba, C.: Activation of glucose-6-phosphate dehydrogenase of enzyme-deficient subjects. II. Properties of the activators and the activation reaction. J. clin. Invest. 40: 611 (1961).
30. Rimon, A.; Ashkenazi, J.; Ramot, B. and Sheba, C.: Activation of glucose-6-phosphate dehydrogenase of enzyme-deficient subjects. I. Activation by stroma of normal erythrocytes. Biochem. biophys. Res. Commun. 2: 138 (1960).
31. Robinson, M. A.; Brown Loder, P. and De Gruchy, G. C.: Red-cell metabolism in non-spherocytic congenital haemolytic anaemia. Brit. J. Haemat. 7: 327 (1961).
32. Saint, E. G. and Hunt, R.: Hemochromatosis with heart failure complicating congenital non-spherocytic hemolytic anemia. Austr. Ann. Med. 4: 105 (1955).
33. Selwyn, J. G. and Dacie, J. V.: Autohemolysis and other changes resulting from incubation in vitro of red cells from patients with congenital hemolytic anemia. Blood 9: 414 (1954).
34. Tanaka, K. R.; Valentine, W. N. and Miwa, S.: Pyruvate kinase (PK) deficiency hereditary nonspherocytic hemolytic anemia. Blood 19: 267 (1962).
35. Tishkoff, G. H.; Robscheit, F. S. and Whipple, G. H.: Red cell stroma in dogs. Variations in the stroma protein and lipid fractions related to experimental conditions. Blood 8: 459 (1953).
36. Valentine, W. N.; Tanaka, K. R. and Miwa, S.: A specific erythrocyte defect (pyruvate kinase) in three subjects with congenital non spherocytic hemolytic anemia. 75th annu. Meet. Ass. amer. Physic., Atlantic City N. J. 1961. Trans. Ass. amer. Phys. 74: 100 (1961).
37. Waller, H. D. and Löhr, G. W.: cit. by Prankerd (27).

Authors' address: Prof. P. Brunetti, Drs. A. Puxeddu, O. Nenci and E. Migliorini, Medical Clinic, Univ. of Perugia (Italy).

Acta haemat. 30 103–110 (1963)

Department of Anatomy School of Medicine, University of Washington,
Seattle, Washington

The Sizes and Interrelations of Lymphocytes in Thoracic Duct Lymph and Lymph Node of Normal and Stimulated Rats

By WILLIAM O RIEKE, N B EVERETT
AND RUTH W CAFFREY

The lymphocytes in thoracic duct lymph and lymph nodes have often been divided on the basis of size into three classes large, medium and small. Unfortunately the limits between these classes have usually been arbitrarily chosen and have varied among species and among authors (*1 2, 3 4 5 6, 7*) The problem of classification by morphology alone has been further complicated by the question of whether to measure a cell's total diameter or its nuclear diameter and by the fact that the size of cells in smears is markedly influenced by technique and the presence or absence of erythrocytes (4 8)

Although the classes of lymphocytes have been established from morphological criteria alone, the demonstration that the developmental sequence of lymphocytes is from large to medium to small (8) has provided the suggestion that the classes may also be separated on the basis of their proliferative behavior If possible, it would be desirable to have each class of lymphocytes include only those cells which exhibit similar developmental patterns. By using tritiated thymidine (TTH) with radioautography the rates of proliferation and labeling patterns of lymphocytes may be studied in relation to their cell or nuclear diameters, and the classes of large, medium and small may be defined from these functions. A major purpose of the present work is to classify lymphocytes so that the members of a given class have similar proliferative behavior and to establish morphologic boundaries between the classes in such a

manner that the similarities in development are preserved. A further purpose is to present evidence of changes in cell size induced by stimulation with irradiation or pertussis vaccine and to set the limits between the classes of lymphocytes so as to include these changes.

Previous papers from this laboratory have presented data showing that small lymphocytes may be grouped with respect to circulating life span into long and short lived types (9 10). The long lived small lymphocytes have been shown to constitute the majority of the population in thoracic duct lymph (TDL) and lymph node (10) and have been strongly implicated in immune responses. When small lymphocytes are considered herein, it is this group that is being discussed.

Materials and Methods

Male rats (60 to 200 gm.) of either the Lewis or Sprague-Dawley strain were used in all experiments. Thoracic duct lymph was collected and smears of lymph or minced mesenteric lymph node were made for radioautography employing standard techniques previously described (5). Rats were labeled with tritiated thymidine (Schwarz Biochemicals, M Vernon, N Y specific activity 1.9 c./mM) given according to one of the following schedules

1 Single injection. TTH in dose of 1 μc./gm. body weight was given intravenously to 4 animals which were sacrificed 15 minutes later. This schedule labeled only those cells synthesizing deoxyribonucleic acid (DNA) in preparation for division.

Intravenous infusion. TTH in dose of 1 μc./gm. body weight was injected intraperitoneally in 6 animals every 4 hours for 36 hours. The animals were sacrificed 4 hours after the last injection. This schedule was found to be equivalent to continuous infusion of TTH for 36 hours and labeled 100% of all rapidly proliferating cells.

3 Multiple injection interval. Four animals received 0.5 μc./gm. body weight TTH in series of 12 injections during 16-day period. The animals increased in weight from an average of 60 140 gm. during the injection period. Two weeks were allowed after the last injection in order to clear label from the rapidly proliferating elements and leave only the slowly proliferating elements labeled. This schedule proved valuable in labeling many of the long-lived small lymphocytes of TDL and lymph nodes and in studying the relationship of these cells their large and medium precursors under normal conditions.

Pertussis vaccine in dose of 0.2 ml. 5 10^9 cell ml.) or total body gamma irradiation 50) from Co^{60} source was used to induce changes in lymphocyte size and proliferation. Animals labeled with TTH according to the multiple-injection-interval schedule were given pertussis or irradiation at the end of the two-week period after labeling and then were sacrificed from 1 to 14 days after stimulation.

After radioautographic processing smears of lymph or lymph node were evaluated microscopically and cell and nuclear diameters were measured directly by means of a calibrated ocular micrometer. Because large cells tended to be found more frequently at the end of smears, each slide was systematically examined by scanning along the middle of the smear from one end to the other counting every cell which touched the center mark of the micrometer. Further details on measurements [illegible] and Dorm-

Results and Discussion

Smears from TDL or mesenteric node showed that cells ranged in size from 5 to 20 microns across. When labeling patterns were considered, it became evident that total cell diameter was not a reliable means of classifying cells into groups which showed similar proliferative behavior. There was a particular lack of reliability in stimulated rats where an increase in the size of both cells and nuclei was accompanied by a decrease in nuclear-cytoplasmic ratio. Accordingly a classification based on nuclear diameters and called "nuclear index" was developed and found to be reliable. The term nuclear index is defined as that product (expressed in square microns, μ^2) obtained when the nuclear diameter of a cell measured

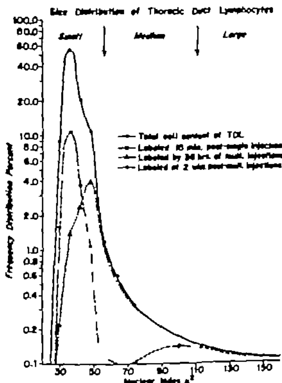


Fig. 1. The normal size distribution of thoracic duct lymphocytes in the rat is shown in relation to the sizes of lymphocytes labeled by various injection schedules of tritiated thymidine. Note that   logarithmic scale is used on the ordinate in order to represent clearly the small fraction of cells that are large and medium.

in one direction is multiplied by the nuclear diameter measured at right angles to the first. Utilizing the nuclear index, the frequency distribution of the various size cells in the TDL of several unstimulated animals was determined and plotted (fig 1) It is seen that the most frequently encountered cell (56 %) was one with a nuclear index of 36 μ^2 and hence with a nuclear diameter of 6 μ

In establishing the classes of lymphocytes so that each class contained only those cells which showed similar labeling patterns, it was necessary to recall not only that large and medium lymphocytes label immediately with TTH and give rise to smaller cells, but also that there is recent evidence that small lymphocytes may enlarge and leave their category Indeed GOWANS et al (11) and PORTER AND COOPER (12) have shown enlargement of small lymphocytes to large pyroninophilic cells when the small cells were stimulated by antigens from a foreign strain. The data to be presented below show a similar enlargement of small cells within one strain when appropriately stimulated Together these lines of evidence indicate that while the largest and smallest lymphocytes probably fall into the typical developmental scheme noted above, there is an intermediate class of cells (medium) which may arise either by division of the large or enlargement of the small lymphocytes.

From a proliferative point of view then the classes of lymphocytes were established as follows

1 *Large* These are rapidly proliferating cells probably deriving from some "stem" cell in lymph nodes and dividing to form other cells at least many of which are medium lymphocytes. 50 % or more of these cells are labeled 15 minutes after a single injection of TTH 100 % are labeled by intensive injections for 36 hours, and none is labeled two weeks after injections are discontinued All lymphocytes with a nuclear index greater than 81 μ^2 are labeled by 36 hours of intensive TTH injections (fig 1) and could be viewed as belonging to this rapidly proliferating class in the non-stimulated rat However the lower limit of the large must be high enough to exclude the largest cell which arises after stimulation of small lymphocytes. Because heavily labeled cells with a nuclear index as great as 100 μ^2 are occasionally found (fig 2) after irradiation of multiple injection animals which initially contain labeled lymphocytes with a nuclear index no greater than 64 μ^2 the lower limit of large in the rat is set at 110 μ^2

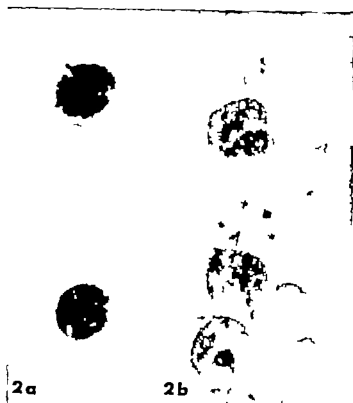


Fig 2. Radioautographs of thoracic duct lymphocytes from control and irradiated rats showing enlargement of lymphocytes with irradiation.

) An example of the largest labeled lymphocyte found in control animal 28 days after multiple injections of tritiated thymidine. × 1500.

b) An example of the largest labeled lymphocyte found in an animal 28 days after multiple injections of tritiated thymidine and 12 days after the administration of 300 total body gamma irradiation. × 1500.

2 *Small.* These are non-dividing cells which do not label 15 minutes after TTH and become labeled only by divisions of labeled precursors. When multiple injection interval animals which contain labeled lymphocytes that are mainly small are stimulated with pertussis vaccine, there is an increase in the average nuclear index of labeled cells from 36 μ^2 to 50 μ^2 (fig 3) Most of these stimulated lymphocytes are still "small" that is, incapable of division as shown by their failure to label with a single TTH injection. A few however may enlarge sufficiently to be considered medium, and it is probable that many more enlarge to medium

when they are stimulated by radiation rather than pertussis (fig 3) When these observations are balanced against the fact that multiple-injection interval animals which may contain a few labeled medium cells have no labeled cells larger than 64 μ^2 the boundary between medium and small is set at 56 μ^2

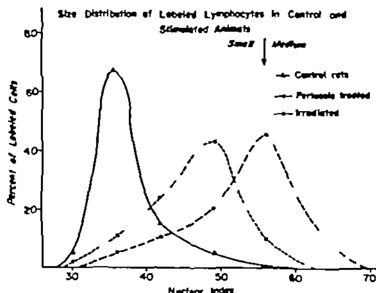


Fig 3. The size distribution of labeled thoracic duct *lymphocytes in control rats* is compared to the size distribution of labeled lymphocytes in rats stimulated with irradiation or pertussis vaccine to show enlargement of small lymphocytes with stimulation. The control curve represents the sizes of labeled lymphocytes found in rats at any time between the 16th and 30th day after multiple injections of tritiated thymidine The pertussis curve represents the sizes found 18 days after multiple injections of tritiated thymidine and after 2 days of pertussis vaccine administration. The irradiation curve shows the sizes found 28 days after multiple injections of tritiated thymidine and 12 days after the administration of 300 total body gamma irradiation.

3 *Medium.* These cells which from the above considerations have a nuclear index of less than 110 μ^2 and more than 56 μ^2 arise either by division of the large or enlargement of the small lymphocytes. They are capable of division as shown by the fact that some are labeled 15 minutes after TTH The difference between this class and the large is seen in intensive-injection rats where even 10 days after 100 % of the large are labeled a small percentage of medium remain unlabeled In addition two weeks after multiple injections all large lymphocytes have lost their radioactivity but a few medium cells remain labeled. It is believed from the present study as well as from the data of GOWANS et al (11) that medium cells which

arise by enlargement of small lymphocytes subsequently divide to form more small cells.

It is hoped that the present classification which provides similarities in development and proliferation within groups will prove useful in reducing confusion in existing systems based only on morphology It is to be noted that the proposed classification depends primarily on proliferative behavior as revealed by DNA labeling patterns and that the nuclear index values which separate classes may not be the same from one species to another

Acknowledgements: The authors wish to thank Prof. MELVIN FIGLEY and ROBERT PARKER of the Department of Radiology University of Washington School of Medicine, for their advice and use of the Co^{60} radiotherapy unit employed in the irradiation of the animals in this study

This work was supported by AEC research grant AT (45-1) 1377

Summary

A classification of the lymphocytes of the thoracic duct and mesenteric lymph node is proposed on the basis of labeling patterns shown with tritiated thymidine and radioautography in control and irradiation or pertussis-stimulated rats. The classification employs a term called nuclear index (the product in square microns of two perpendicular nuclear diameters) and provides similarities in proliferation as well as in appearance within groups of lymphocytes. New evidence is presented to show that small lymphocytes enlarge with at least some becoming medium when appropriately stimulated.

The following kinetic characteristics of each class are developed 1 Large lymphocytes (nuclear index of 110 μ^2 or more) are rapidly proliferating cells which derive from stem cells. 2. Medium lymphocytes (56 to 110 μ^2) probably represent mixed class originating mainly by division of large lymphocytes but also in part by enlargement of small lymphocytes. 3. Small lymphocytes (56 μ^2 or less) are non-dividing cells which become labeled only as consequence of being formed by division of labeled larger precursors.

Résumé

Les auteurs proposent une classification de lymphocytes du ductus thoracicus et des ganglions lymphatiques mésentériaux à la base de leurs recherches à l'aide de la thimidine marquée au tritium et de l'autoradiographie chez des rats normaux irradiés ou traités par un vaccin anti-coqueluche. La classification se base sur un index nucléaire (produit de deux diamètres perpendiculaires en microns carrés) et présente des analogies de la prolifération et de la morphologie parmi certains groupes de lymphocytes. On peut obtenir des *indices nouveaux pour l'existence* d'une croissance des petits lymphocytes, dont au moins quelques-uns atteignent une grandeur moyenne, ils sont stimulés d'une façon adéquate.

Les caractéristiques suivantes de chaque classe sont établies 1 Les grands lymphocytes (index nucléaire 110 μ^2 et d'avantage) sont des éléments d'une prolifération rapide, descendant de cellules-souches. 2. Les lymphocytes moyens (56–110 μ^2) forment probablement une classe mixte, formés surtout par la division de grands lymphocytes, en partie par l'augmentation de petits lymphocytes. 3. Les petits lymphocytes (56 μ^2 et moins) sont des cellules qui ne peuvent plus se diviser et qui sont uniquement marqués parcequ'ils sont formés par la division de formes antécédentes plus grandes et marquées.

Zusammenfassung

Auf Grund von Untersuchungen mit Tritium-markiertem Thymidin und Autoradiographie bei Kontrolltieren und bei bestrahlten oder mit Pertussis-Vakzine geimpften Ratten wird eine Klassierung der Lymphozyten in Ductus thoracicus und mesenterialen Lymphknoten vorgeschlagen. Die Klassierung stützt sich auf den sogenannten Kernindex (das Produkt von zwei zu einander senkrechten Kerndurchmessern in Quadratmikren) und ergibt Ähnlichkeiten der Proliferation und der Erscheinungsform innerhalb gewisser Gruppen von Lymphozyten. Es ergeben sich neue Anhaltspunkte dafür, daß kleine Lymphozyten sich vergrößern, wobei mindestens einzelne mittlere Größe erreichen, wenn sie entsprechend stimuliert werden.

Es lassen sich die folgenden kinetischen Charakteristika jeder Klasse aufstellen. 1. Große Lymphozyten (Kernindex 110 μ^2 oder mehr) sind rasch proliferierende Elemente, die von Stammzellen abstammen. 2. Mittelgroße Lymphozyten (56 bis 110 μ^2) bilden wahrscheinlich eine gemischte Klasse, die vorwiegend durch Teilung großer Lymphozyten, zum Teil aber auch durch Vergrößerung kleiner Lymphozyten entsteht. 3. Kleine Lymphozyten (56 μ^2 und weniger) sind nicht teilungsfähige Zellen, die nur deshalb markiert werden, weil sie durch Teilung markierter größerer Vorstufen gebildet werden.

References

1. McMillan, D. B. and Engelbert, V. E.: The cells of the normal lymph node. Canad. J. Zool. 38: 615 (1960).
2. Little, J. R., Brecher, G.; Bradley, T. R. and Rose, S.: Determination of lymphocyte turnover by continuous infusion of H³-thymidine. Blood 19: 236 (1962).
3. Nossal, G. J. V. and Mäkelä, O.: Genetic aspects of antibody formation. Lab. Invest. 10: 1094 (1961).
4. Schooley, J. C. and Berman, I.: Morphologic and autoradiographic observations of H³-thymidine-labeled thoracic duct lymphocytes cultured in vivo. Blood 16: 1133 (1960).
5. Everett, N. B.; Rieke, W. O.; Reinhardt, W. O. and Yoffey, J. M.: Radioisotopes in the study of blood cell formation with special references to lymphocytopoiesis. Ciba Foundation Symposium on Haemopoiesis (Churchill, London 1960).
6. Bryant, B. C. and Kelly, L. S.: Autoradiographic studies of leukocyte formation. Proc. Soc. exp. Biol., N.Y. 99: 681 (1958).
7. Porter, K. A. and Cooper, E. H.: Recognition of transformed small lymphocytes by combined chromosomal and isotopic labels. Lancet i: 317 (1962).
8. Everett, N. B.; Reinhardt, W. O. and Yoffey, J. M.: The appearance of labeled cells in the thoracic duct lymph of the guinea-pig after administration of tritiated thymidine. Blood 15: 82 (1960).
9. Caffrey, R. W.; Rieke, W. O. and Everett, N. B.: Radioautographic studies of small lymphocytes in the thoracic duct of the rat. Acta haemat. 28: 145 (1962).
10. Everett, N. B.; Caffrey, R. W. and Rieke, W. O.: The small lymphocyte of the rat: Rate of formation, extent of recirculation and circulating life span. Proc. 9th int. Congr. Haemat., Mexico City 1962 (in press).
11. Gowans, J. L.; McGregor, D. D.; Cowen, D. M. and Ford, C. E.: Initiation of immune responses by small lymphocytes. Nature 196: 651 (1962).
12. Porter, K. A. and Cooper, E. H.: Transformation of adult allogenic small lymphocytes after transfusion into newborn rats. J. exp. Med. 115: 997 (1962).

Author's address: Dr. W. O. Rieke, Dr. N. B. Everett and Mrs. Ruth W. Caffrey, Dept. of Anatomy, Univ. of Washington, Seattle 5, Wash. (USA)

Acta haemat. 30 111 122 (1963).

Department of Pathology Karolinska Institutet, Stockholm
(Director: Prof. B. Thorell)

The Histogenesis and Haematology of Virus-Induced Myeloid Leukaemia in the Fowl*

By Bengt Lagerlöf and Pär Sundelin

A number of studies have been made in the past on the histogenesis and development of virus-induced erythroleukaemia in the fowl (1 3 5 8 9 10) Myeloid leukaemia, on the other hand, has received less attention in this respect in spite of the many virological and biochemical investigations performed regarding this disease. In the myeloid leukaemia, the granulopoietic stem cells located extravasally in the bone marrow undergo neoplastic transformation. In contrast to the case in erythroleukaemia, there is a considerable age variation in respect of the susceptibility to myeloid leukaemia virus. Newly hatched chicks are highly sensitive and display a high frequency of leukaemia, two week-old chicks are slightly less sensitive, while at 2 months the resistance to the virus is complete (7 11) Another difference from erythroleukaemia virus is that the myeloid virus in the plasma preparations employed and under the prevalent laboratory conditions displays a wider cytotropism for not only leukaemia but also visceral lymphomatosis, renal tumours, osteopetrosis and haemangiomatosis may develop in chicks inoculated with the virus at 2 weeks of age (2, 7 11) Some animals with leukaemia run an atypical course of the disease and develop a subleukaemic anemia characterized by severe anaemia of haemolytic type, granulocytopenia and subleukaemic blood picture (7)

The purpose of the present study was to establish the histogenesis and haematological development of the myeloid form of leukaemia and is intended to constitute a basis for further investigations into the disease.

* A grant has been received for this research from the Lady Tata Memorial Trust.

Material and Methods

Fowl strain: A white Leghorn (Edo) strain was used. The properties and maintenance of this strain have been reported earlier (5, 7)

The *virus strain* and preparation of virus suspensions have been described elsewhere (7 11) Each chick was given 0.3 ml. heparinized plasma from leukaemic chicks; according to adenosine triphosphatase values these doses were expected to give at least 90% takes in newly-hatched chicks.

Haematologic values: Regular determinations were made of the haematocrit, erythrocytes and leukocytes (concentration and differential counts). T minimize the volume of the blood required from these small chicks method was evolved in which all the above values could be read from the haematocrit and smears of the peripheral blood. I separate experiment the ratio between haematocrit and the erythrocyte concentration was determined with gradually increasing dilution of blood in autologous plasma (fig. 1). *The haematocrit was determined in the usual way in heparinized capillary tubes,* which were centrifuged for 5 minutes in haematocrit centrifuge (International). For the cell counts 10 to 20 cu. mm. of blood were diluted in 10 ml. of Ringer' solution. The counts covered 8 small squares of Fuchs-Rosenthal counting chamber corresponding to 0.1 cu.mm., or at least 200 cells.

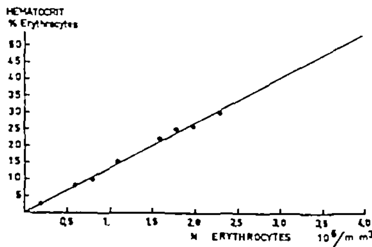


Fig 1 The correlation between the erythrocyte concentration in the haematocrit and the erythrocyte count with increasing dilution.

The *number of erythrocytes per cu.mm.* of blood was then interpolated from the graph in fig. 1 giving the ratio between the haematocrit and erythrocyte concentration.

The leukocyte count was difficult to perform in suspension, owing to the preponderance of erythrocytes, which being nucleated cannot be eliminated by haemolysis. The leukocytes were therefore estimated by calculating the number of white cells per 6000 to 7000 *erythrocytes in smears. The coefficient of variation of this estimate of whit* corpuscles was 13%

T ascertain whether the sample-taking had any appreciable effect on the haematologic values, and to est the reproducibility of the haematocrit, determinations of haemoglobin and haematocrit were made on alternate days on control group of 10 one-

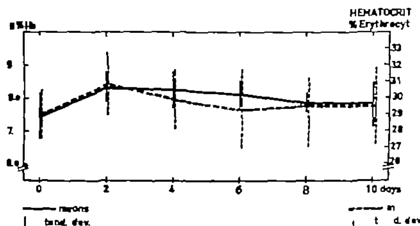


Fig. 2. The relationship between the haematocrit and haemoglobin in control chicks as determined by repeated blood samples.

week-old chicks. For the former test 20 cu.mm. of venous blood were haemolyzed in 3.5 ml. of 0.1% sodium carbonate, and the extinction of the haemoglobin solution at 5400 Å was measured in a Lineon 3 photometer. The relationship between the variation in haematocrit and haemoglobin is illustrated in fig. 2. The blood loss due to the sampling, which is of the same order as the amount taken for the samples in the main experiments, proved to be negligible.

Histologic preparation. From the chicks on which a histologic examination was to be performed the marrow in both femurs and tibias was removed and specimens were taken of the liver, spleen, kidneys and lungs. The specimens were fixed in Stieve's fluid (mercuric chloride, formalin and glacial acetic acid) and stained by the haematoxylin-eosin and Giemsa methods. The marrow sections were also stained by Van Gieson's technique, and sections from the spleen also by Hueck's method for iron pigment.

Results

Haematologic Findings

The development and course of the myeloid leukaemia in the peripheral blood was examined in a group of 9 five-day-old chicks. The results are shown in figs. 3–5.

Leukaemia cells. The onset of leukaemia occurred at widely varying periods after virus inoculation (fig. 3). In one chick leukaemia cells were recognized in the peripheral blood after only 6 days and the disease progressed until death on day 18 (no. 4). The course was similar for some of the other chicks except for a slightly later onset (nos. 2, 5 and 6). Two chicks exhibited short episodes of leukaemia about days 22 and 26, but the ordinarily seen progressive course of the disease did not start until days 43 and 61 respectively

Fig 3. Leukaemia cell infiltration in the blood of chicks after intravenous inoculation of myeloid leukaemia virus at 5 days of age.

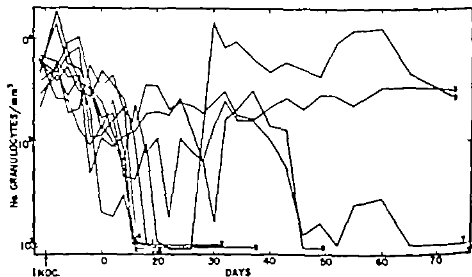


Fig 4. Granulocytes in the blood of the same chicks as in fig. 3.

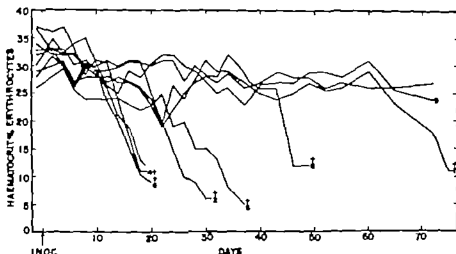


Fig. 5. Haematocrit values in the same chicks as in fig. 3. The crosses indicate the death of the chicks.

(nos. 7 and 8) One chick had mild transient leukaemia for 2 weeks and thereafter no signs of the disease (no. 9) Another chick also had peripheral leukaemia of brief duration but this was accompanied by rapidly progressing anaemia, and death from "subleukaemic anaemia" (7) followed on day 19 (no. 1)

Granulocytes. Several of the chicks showed a slight decrease in the granulocyte counts during the first 2 weeks (fig. 4) At a late stage, when there was evident peripheral leukaemia, severe granulocytopenia often developed (e.g. nos. 2 4 6 and 8) One of these chicks also showed a drop in the granulocyte count in connection with an episode of peripheral leukaemia on about day 30 (no. 8) A similar course was noted for a chick recording two episodes of peripheral leukaemia on about day 26 and 52 (no. 7) In one case transient granulocytosis developed rapidly after a drop in connection with peripheral leukaemia on days 20 and 52 (no. 9)

Erythrocytes. All the chicks that died showed a marked decrease in the erythrocyte concentration (fig. 5) Some chicks exhibited a slight decrease during the first week but in all except two survivors (nos. 3 and 9) there was a steep drop one or two weeks prior to death. The two survivors both showed a transient fall about day 22, and in one of them this was associated with mild leukaemia between

days 20 and 3? (no. 9) The other survivor did not display a leuk aemic blood picture (no. 3) These two chicks were observed for a further 4 months, during which time the blood values were normal, with no further signs of leukaemia and no renal tumours or lymphomatosis.

Histologic Findings

The main results of the histologic examination are presented in table I Six chicks, inoculated at 3 days of age, were examined daily from day 2 to 9 and on days 11 and 13 The chicks to be sacrificed were selected at random irrespective of any leukaemic blood picture. Chicks showing no signs of leukaemia have not been included in the table I

As far as the leukaemia is concerned, the target organ for the virus is the bone marrow and the first neoplastic signs were found there. The granulopoiesis occurs in the extrasinusoidal tissue and the first morphologic signs of the leukaemia were multiple small extra sinusoidal foci in the femur marrow These foci, which were first observed on the day 4 consisted of atypical myeloid tumour cells among which few granulocytes were noted (fig 6) A frequent feature was the invasion of the sinusoids by leukaemic cells (fig 7) The small leukaemic foci extended and formed tumour buds (fig 8) which merged to produce larger foci and finally massive invasion of the marrow The marrow of the tibia, on the other hand which contains a larger amount of fat, displayed no early changes, but at a late stage of the disease it often contained small leukaemic foci between regions of massive leukaemic invasion (fig 9)

To judge from the histologic findings there was during the first 3 or 4 days after inoculation a general reaction in the marrow consisting in hyperplasia of both granulopoietic and erythropoietic tissue No cell counts were performed however There was no appreciable lymphoid hyperplasia such as was found in the case of subleukaemic anaemia (7) At the later stage of leukaemia there was a replacement of the erythropoietic cells by leukaemic tissue and in massively invaded marrow erythropoiesis was practically absent.

Leukaemic infiltration in extramedullary organs was found already one day after the bone marrow involvement. The distant infiltration was mild at first, with isolated leukaemia cells and small

Table I

Leukaemic cell infiltration in different organs after intravenous inoculation of leukaemia virus.

Days after inoc.	Grade of leukaemic infiltration				
	Bone marrow	Spleen	Liver	Kidneys	Lungs
4	+				
4	+	—	—	—	—
4	++	—	—	—	—
5	+		+	—	—
5	+	++	—		+
5	++	++	—		
6	+	—	+		
6	++	—	++	+	+
6	+++		+		+
6	+++	—	++		+
7	+	++	—		+
7	+++	++	+		+
7	+++	+++	++	++	++
7	+++	+++	++		
7	++++	+++	++	++	+ +
8	++	++	—	—	++
8	+++	—	+	—	+
8	+++	++	++	+	+
9	+	++	+	—	+
9	+		+	—	—
9	++	—	+	—	+
9	++	—	+	+	+
9	++++	++	+	+	++
11	+++	++++	+	++	++
11	+++	+++	+	++	++
11	+++	+++	++	+++	++++
11	++++	++++	+++	+++	+++
15	++				
15	+++	++++	+++	+	+++
15	+++	++++	+++	+	+++
15	++++	++++	+++	++	+++
15	++++	++++	+ +	+++	+++

Leukaemic infiltration in

Bone marrow:

- \+ Occasional minute foci of leukaemia cells as shown in figs. 6 and 7
- ++ Many small, limited foci of leukaemia cells.
- +++ Several large, partly confluent foci of leukaemia cells.
- ++++ Heavy leukaemic infiltration with almost complete replacement of the normal tissue.

Spleen, liver and lung

- \+ Occasional leukaemia cells.
- ++ Several leukaemia cells, often in small foci in the spleen and liver
- +++ Marked leukaemic infiltration with several foci of leukaemia cells in the spleen and liver
- ++++ Heavy leukaemic infiltration.

foci At a later stage the spleen, liver and kidneys contained confluent infiltrates, and in the terminal stage the greater part of the spleen and liver were involved. The only remaining normal tissue in the spleen was found in parts of the Malpighian corpuscles, while in the liver only central parts of the lobuli were saved (7) The kidneys displayed consistently less marked infiltration which for the most part occurred as foci in the cortex

In the lung tissue the infiltration was more diffuse but in the terminal stage it could be almost massive and completely obliterate the normal architecture

In contrast to the findings in the subleukaemic anaemia described earlier (7) no iron pigment was found in sections from the spleens.

Non-specific changes A control group of 42 three-day-old chicks were inoculated with 0.5 ml of leukaemia plasma which had been inactivated for 48 hours at 37 C. None of 6 chicks left for 3 months to test the effect of inactivation developed leukaemia. After one to six days one group of 6 of the remaining chicks was sacrificed daily and examined histologically in the same way as the virus-inoculated chicks In most of these the marrow displayed a non-specific myeloid reaction that was morphologically distinct from leukaemic proliferation in that it occurred diffusely in the marrow and that the cell flora was dominated by mature granulocytes with small interspersions of myeloblasts (fig 10) As in the case of the virus inoculated chicks the erythropoiesis was also slightly stimulated

---

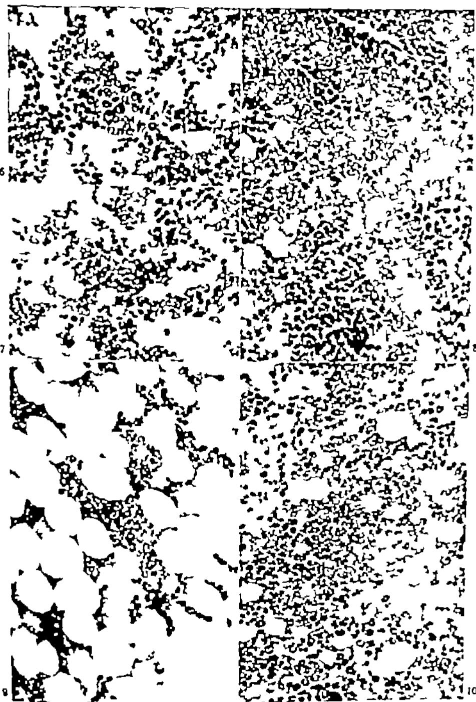
Fig [illegible] Femur marrow of chick 4 days after inoculation with virus. An early leukaemic focus seen in the extrasinusoidal tissue (centre Haematoxylin and eosin. × 400.

Fig 7 Femur marrow of the same chick in fig. 6. I[illegible]va ion of leukaemia cells into sinusoid from minute leukaemic focus. Giemsa. 400.

Fig 8 Femur marrow of chick 5 day after inoculation with virus. Leukaemic tumour bud proliferating in the extrasinusoidal tissue few granulocytes are seen. Within the sinusoids the number of erythropoietic cells is normal. Giemsa. 400.

Fig 9 Tibia marrow of chick 6 day fter inoculation ith irus. Intra- and extrasinusoidal leukaemic f in he f marrow There is no evidence of normal haematopoiesis Giemsa 400

Fig 10 Femur marrow of chick 3 days af er inoculation w h inact vated leukaemic plasma. There is ac n granulopoiesis, domina ed by mature granulocytes, mong which re few myeloblasts Haematoxyl and eosin. 400.


6
7
8
9
10

Discussion

It has been shown in earlier studies that virus-induced erythroleukaemia in the fowl begins as small intrasinusoidal foci in the marrow unlike the diffuse hyperplasia seen in the erythroid reaction, for instance after haemolysis. The first signs of erythroid neoplasia were found on the third day after inoculation and on day 5 there was peripheral infiltration The erythroleukaemia followed a rapid course and the chicks died only 7 days after inoculation (5 8, 9)

To judge from the findings of the present study myeloid leukaemia in the fowl has in principle a similar histogenesis and development After a lag phase of 3 days the first morphologic signs of myeloid neoplasia were found extrasinusoidally in the haematopoietic marrow of the femur (table I fig 6) These small tumour foci often displayed signs of invasion into the sinusoids (fig 7) and thus created the conditions for early haematogenic spread of the tumour cells The extramedullary infiltration also took place considerably earlier in myeloid leukaemia than in erythroleukaemia as judged from the extent of the leukaemic proliferation in the marrow In erythroleukaemia no distant infiltration is seen until the leukaemia is advanced in marrow (5) while in myeloid leukaemia such infiltration is evident when the marrow is only slightly involved. This migh be due to the capacity of active mobility of the myeloid cells.

The duration of the leukaemia, from the first signs of infiltration of the peripheral blood until the death of the chick varied from 1? to 15 days (fig 3) the longer survival times include aleukaemic periods. The duration of the period of final progressive leukaemia ranged from 6 to 15 days for the chicks in fig 3 The slightly shorter survival period in erythroleukaemia (6) is probably due to the fact that chicks with myeloid leukaemia do not die until the leukaemia is considerably more advanced and need not imply a major proliferative activity of the erythroleukaemia

In contrast to myelosis in man (4) the myeloid leukaemia in the fowl displays a tendency to focal peripheral infiltration located for instance periportally in the liver Th erythroid leukaemia in the fowl has a considerably more diffuse pattern of infiltration than the myeloid form

4. Goldberg, G. M. and R[illegible]stone, A. J. A study of malignant lymphomas and leukaemias. II. A diagnostic approach to lymphogenous leukaemias and [illegible] lymphomas (with reference to the involvement of the lymphatics). Cancer 13, 520–527 (1960).
5. Lagerlöf, B. Studies of fowl erythroleukaemia induced by intact cells of [illegible] I. The fate of transplanted erythroleukaemia cells, as revealed by histo-pathological and quantitative cytological investigations. Acta Path. microbiol. scand. 49, 433–434 (1960).
6. Lagerlöf, B. Studies of fowl erythroleukaemia induced by intact cell or [illegible] II. Response differences. Acta Path. microbiol. scand. 49, 45[illegible]–466 (1960).
7. Lagerlöf, B. and Sundelin, P. Variations in the pathogenic effect of [illegible] leukaemia [illegible]. Acta Path. microbiol. scand. (in press).
8. Pontén, J. Studies on [illegible] erythroblastosis [illegible]. J. Morphologic events after induction by large dose of erythroblastosis [illegible]. Acta Path. microbiol. scand. 47, 81–93 (19[illegible]).
9. Pontén, J. and Thorell, B. The histogenesis of [illegible]-induced [illegible] leukaemia. J. Nat. Cancer Inst. 18, 443–453 (1957).
10. Ratcliffe, H. L. and F[illegible]rth, J. Studies of the pathogenesis of erythroleukosis. Amer. J. Path. 9, 16[illegible]–184 (1933).
11. Thorell, B. Modifications of the host reaction towards leukaemia [illegible]. Proc. VIIth Congr. Int. Soc. Haematology pp. [illegible]82–[illegible]92 (19[illegible]).

[illegible] address: Drs. B. Lagerlöf and P. Sundelin, Dept. of Pathology, Karolinska I[illegible] [illegible], Sweden.

Acta haemat. 30, 123–127 (1963)

From Medical Department C, Copenhagen County Hospital, Glostrup
(Chief Dr P From Hansen)

Histochemical Studies of Leukocytes from an Inflammatory Exudate

IV Uridine Diphosphate Glucose Glycogen Transglycosylase

By Henrik R. Wulff

In a previous study it was shown that the glycogen content of neutrophilic granulocytes and macrophages from skin windows increases with the progress of the inflammatory process (8) The migrating cells also presented an increasing phosphorylase activity and it was assumed that leukocyte glycogen is synthesized from glucose 1 phosphate via the phosphorylase system. In other tissues, however glycogen synthesis has been shown to take place from uridine diphosphate glucose by uridine diphosphate glucose–glycogen transglycosylase (5 7) and in the present investigation this alternative pathway for the biosynthesis of glycogen has been studied histochemically in migrating leukocytes.

Methods

Principle of histochemical reaction (7) The leukocytes are incubated in medium containing uridine diphosphate glucose and a small amount of glycogen as primer During incubation cells possessing sufficient uridine diphosphate glucose glycogen transglycosylase activity synthesize polysaccharide in the cytoplasm, which is afterwards demonstrated by staining with iodine. Addition of glucose-6-phosphate has been show to accelerate the reaction in other tissues.

Sampling of leukocytes and histochemical procedure The leukocytes were obtained by the "skin window technique, as previously described (6, 8). Six skin window" experiments were made on two healthy subjects, and the coverslips were changed hourly for 12 hours. Each coverslip with migrating leukocytes was dried for few seconds after removal from the lesion, flooded with 3 drops of incubation medium and left for 2 hours at room temperature in moist chamber After incubation the coverslip preparation was dipped in distilled water dried quickly in air and fixed in methanol. Finally the preparation was stained for one minute with (undiluted) *Gram's* iodine solution and mounted in glycerine jelly containing 20% *Gram* solution (or the preparation as

Aided by grant from Arvid Nilsson's Fond"

4. Goldberg, G. M. and Rittenhouse, A. J. A study of malignant lymphomas and leukaemias. II. A diagnostic approach to lymphogenous leukaemias and myelogenous leukaemias (with reference to the involvement of the lymphatics). Cancer 13: 520-527 (1960).
5. Lagerlöf, B. Studies of fowl erythroleukaemia induced by intact cells or virus. I. The fate of transplanted erythroleukaemic cells, as revealed by histo-pathological and quantitative cytological investigations. Acta Path. microbiol. scand. 49: 433-434 (1960).
6. Lagerlöf, B. Studies of fowl erythroleukaemia induced by intact cells or virus. II. Response differences. Acta Path. microbiol. scand. 49: 455-466 (1960).
7. Lagerlöf, B. and Sendelin, P. Variations in the pathogenic effect of myeloid leukaemia virus. Acta Path. microbiol. scand. (in press).
8. Ponten, J. Studies on ... erythroleukaemia. I. Morphologic events after induction by ... large dose of erythroblastosis virus. Acta Path. microbiol. scand. 47: 81-95 (1959).
9. Ponten, J. and Thorell, B. The histogenesis of virus-induced chicken leukaemia. J. Nat. Cancer Inst. 18: 443-453 (1957).
10. Ratcliffe, H. L. and Furth, J. Studies of the pathogenesis of erythroleukosis. Amer. J. Path. 9: 165-184 (1933).
11. Thorell, B. Modifications of the host reaction towards leukaemia virus. Proc. VIIth Congr. Int. Soc. Haematology, pp. ... 39... (1958).

Author address: Drs. B. Lagerlöf and P. Sendelin, Dept. of Pathology, Karolinska Institutet, Stockholm, Sweden.

sion of glucose-6-phosphate from the medium did not alter the reaction appreciably The control preparations, which were incubated in media without uridine diphosphate glucose were negative No uridine diphosphate glucose-glycogen transglycosylase activity was demonstrated in leukocytes from blood films.

Comment

The experiments showed that migrating neutrophils and macrophages are able to synthesize polysaccharide from uridine diphosphate glucose and not only as previously demonstrated from glucose 1 phosphate by the phosphorylase system (8) Both histochemical reactions result in the formation of iodophilic polysaccharides, but notable differences were observed in the distribution of the two enzyme systems and the staining properties and localisation of the histochemically synthesized polysaccharides. Uridine diphosphate glucose-glycogen transglycosylase activity was equally prominent in macrophages and neutrophils, whereas phosphorylase activity was much more intense in neutrophils than in macrophages. The polysaccharides synthesized from glucose 1 phosphate usually stained blue with iodine, indicating an unbranched amylose type of polysaccharide, although "branching enzyme" activity was shown by the occasional occurrence of a violet or brown staining reaction. The uridine diphosphate glucose–glycogen transglycosylase reaction, however resulted in the formation of a polysaccharide, which stained red brown like naturally occurring glycogen in other tissues. Preformed glycogen in leukocytes from peripheral blood and "skin windows" does not stain with iodine under normal conditions, but iodophilic glycogen has been demonstrated in neutrophils from blood in infective states and in neutrophils from purulent exudates (1) The intracellular localisation of the polysaccharides synthesized in the two reactions also differed The polysaccharide formed from uridine diphosphate glucose was localised in smaller and larger masses, whereas the polysaccharide formed from glucose 1 phosphate was usually seen as a diffuse staining of the cytoplasm.

The fact that no uridine diphosphate glucose-glycogen transglycosylase activity was demonstrated in leukocytes from most of the early "skin window" preparations and in leukocytes from blood films is not evidence that they do not synthesize polysaccharide via this pathway A high activity of the enzyme is probably necessary

for its histochemical demonstration since the accumulation of the polysaccharide in the cytoplasm reflects a balance between synthesis and diffusion into the medium In the present experiments poly vinyl pyrrolidone was found to be an essential constituent of the medium. Addition of this inert substance increases the viscosity of the mixture and may thereby diminish the loss of histochemically synthesized polysaccharide by diffusion (2)

It has been suggested that glycogen is normally synthesized from uridine diphosphate glucose, and that the phosphorylase pathway under physiological conditions is concerned with glycogen degradation (4) Histochemical experiments, however have revealed a reciprocal relationship between phosphorylase and uridine diphosphate glucose–glycogen transglycosylase activity in individual muscle fibres (3) and it seems more likely that the biosynthesis of glycogen takes place via both pathways.

Summary

Migrating leukocytes obtained by the "skin window" technique were examined for activity of uridine diphosphate glucose glycogen transglycosylase, using Takeuchi and Glenner method. A positive reaction was observed in both neutrophils and macrophages from the later stages of the inflammatory process. The polysaccharide synthesized from uridine diphosphate glucose stained red-brown with iodine and was localized in smaller and larger masses in the cytoplasm.

Résumé

Etude de l'activité de transglycosylase du système glucose diphosphate de l'uridine glycogène de leucocytes émigrés obtenus à l'aide de la méthode de la fenêtre cutanée, par la méthode de Takeuchi et Glenner. Les neutrophiles, aussi bien que les macrophages des stades tardifs du processus inflammatoire présentent une réaction positive. Les polysaccharides, synthétisés à partir du glucose diphosphate de l'uridine, sont colorés en rouge-brun par le jode et sont localisés en quantités plus ou moins grandes dans le cytoplasme

Zusammenfassung

Ausgewanderte Leukozyten, die mi der «Hautfenster Technik» gewonnen wurden, wurden mit der Methode von Takeuchi und Glenner auf die Aktivität der Uridin-diphosphat-Glukose–Glykogen-Transglykosylase untersucht. Eine positive Reaktion fand sich sowohl in Neutrophilen als auch in Makrophagen von Spätstadien des Entzündungsprozesses. Die aus Uridin-diphosphat-Glukose synthetisierten Polysaccharide färbten sich mit Jod rotbraun und waren in kleineren und größeren Massen im Zytoplasma lokalisiert

References

1. Ehrlich, P. Über das Vorkommen von Glykogen im diabetischen und im normalen Organismus. Z. klin. Med. *6*, 33 (1883).
2. Eränkö, O. and Palkama, A. Improved localization of phosphorylase by the use of polyvinyl pyrrolidone and high substrate concentration. J. Histochem. Cytochem. *9*: 585 (1961).
3. Hess, R. and Pearse, A. G. E. Dissociation of uridine diphosphate glucose glycogen transglycosylase from phosphorylase activity in individual muscle fibers. Proc. Soc. exp. Biol., N. Y. *107*: 569 (1961).
4. Larner, J. Genetic and hormonal control of glycogen metabolism. Fed. Proc. *19*: 971 (1960).
5. Leloir, L. F.; Olavarría, J. M.; Goldemberg, S. H. and Carminatti, H. Biosynthesis of glycogen from uridine diphosphate glucose. Arch. Biochem. Biophys. *81*: 508 (1959).
6. Riis, P. and Wulff, H. R. Dynamic studies of the relation between intravascular and inflammatory neutrophils in normal subjects. Acta haemat. *23*: 276 (1960).
7. Takeuchi, T. and Glenner, G. G. Histochemical demonstration of uridine diphosphate glucose – glycogen transferase in animal tissues. J. Histochem. Cytochem. *9*: 304 (1961).
8. Wulff, H. R. Histochemical studies of leukocytes from an inflammatory exudate. Glycogen and phosphorylase. Acta haemat. *28*: 86 (1962).

Author's address: Dr. H. R. Wulff, Medical Dept. C, Copenhagen County Hospital, Glostrup (Denmark).

Libri

John E. Moseley, **Bone Changes in Hematologic Disorders.** (Roentgen Aspects) Grune & Stratton, New York and London 1963. 231 p., price $ 9.50.

This book, Mount Sinai Hospital Monograph, is collection of typical and atypical bone findings in various blood dyscrasias with excellently reproduced illustrations and brief explanatory text and comments. Each chapter has list of references, mostly from the American literature.

G. Rosenow New York, N.Y

Beutler E., Fairbanks V F and Fahey J L., **Clinical Disorders of Iron Metabolism.** Grune & Stratton, New York and London 1963. 267 p., price $ 8.75.

According to the preface this book is written for the student, the resident and the practising physician. After an interesting but rather lengthy history about iron in medicine (with cartoonlike illustration showing the mountain road to knowledge) single chapters deal aptly with the metabolism of iron, iron deficiencies and their clinical syndromes. Here is also far fetched description of Dibola included an edematous depigmentation syndrome, endemic in the Congo and other parts of Central Africa although (according to the text) "the role of iron lack in this syndrome is far from conclusive —Other chapters present oral and parenteral iron therapy iron poisoning, particularly in children and section on hemochromatosis. Each chapter has an up to date list of references.

G. Rosenow New York, N.Y

Bothwell, Thomas H and Finch, Clement A., **Iron Metabolism.** Little, Brown and Company Boston 1962. 440 p. price $ 15.00.

This is an excellent presentation of all facts of Iron metabolism. Two chapters survey critically the technical methods employed in studies of iron metabolism. It is evident that some of the widely accepted concepts concerning iron metabolism were based on techniques which were "not adequate by present day standards Everyone who is worker in this field will read and study these chapters with particular interest. The other sections on body iron turnover plasma transferrin, ferrokinetics, erythrokinetics, tissue iron stores, iron deficiency anemia and the pathologic and clinical aspects of iron overload are very informative, clearly written presentations with extensive lists of references. The book is highly recommended.

G Rosenow New York, N Y

Kallós P und Waksman, B. H **Progress in Allergy** Vol. 6, S. Karger AG, Basel/New York, 1962. XII + 600 p., 78 ill. 38 tab., price sF 98.

Der neueste Band über die Fortschritte der Allergie bringt vorwiegend Übersichten über immunologische Probleme. Oudin behandelt die theoretischen Grundlagen sowie die neuesten technischen Verbesserungen seiner Gel-Diffusions-Methode. Eine Zusammenfassung über das gruppenspezifische Serum-System (Gc-System) gibt Hirschfeld. Good und Mitarbeiter berichten über immunologische Mangelkrankheiten, ferner Waldenström über Gammopathien. Simonsen und Amos geben je eine ausführliche Übersicht über die Reaktionen gegen Transplantate. Es folgt eine kurze Darstellung über die Eigenschaften der Slow Reacting Substance von Brocklehurst und schließlich behandeln Thorbecke und Benacerraf Zusammenhänge zwischen RES und immunologischen Phänomenen.

G. Ziegler, Basel

Acta haemat. 30 129–137 (1963)

From the Institute of Medical Pathology University of Modena
(Director Professor E. Storti)

RNA and Protein Synthesis in Normal Peripheral Mononuclear Leukocytes

A Radioautographic Study

By U Torelli, G Grossi T Artusi and G Emilia

In recent years, several studies have led to re-examine the problem of the functional capacity of normal peripheral mononuclear leukocytes. According to the results of well tried experiments in several laboratories through out the world peripheral mononuclear cells have the capacity of growing in tissue culture (15) Further more some excellent experiments have proved that transfused leukocytes from normal blood are capable of repopulating the bone marrow of lethally irradiated animals (8, 18) and that circulating blood contains a mobile pool of cells able to initiate immunological reactions, i e. homograft reactions (7)

It seems, therefore, that many peripheral mononuclear leukocytes are cells in a state of "no cell cycle —to use the words of Lajtha et al (11)—capable of passing from an inactive phase (24) to an active one, and thus of starting a new growth cycle Since information about cells in this kind of functional state is particularly needed, mainly in consideration of the general problem of the "stem cell" we thought that the study of some metabolic features of peripheral mononuclear cells might be a useful approach.

Our present knowledge allows to think that among the meta bolic cell processes related to cell differentiation, protein synthesis is the process mainly concerned. As a matter of fact both RNA and proteins are synthesized continuously in a great number of cells, even in the cells of adult connective tissue (4 12) However the quantitative correlation between the two processes has not yet been studied extensively As far as the cells of the blood forming organs

are concerned a direct relationship has been found between RNA synthesis and protein synthesis in differentiated cells of human bone marrow (17)

Nevertheless, a relationship of this type cannot be considered as the rule As it has been pointed out by RAACKE (19) no constant relationship can be observed between the protein synthesis and the amount of RNA present in the cell, the amount synthesized in the cell or even the amount metabolized in the cell. It seems, therefore, conceivable that if a quantitative dissociation between RNA synthesis and protein synthesis can be observed there are differences, as far as the RNA synthesis/protein synthesis ratio is concerned, among blood cells at different levels of functional differentiation. It is quite evident that such study can be performed only on cells, and that autoradiography with tritium labelled compounds is the best method for this purpose

This paper deals with a comparative investigation of RNA and protein synthesis in peripheral mononuclear cells using H^3 cytidine and H^3 leucine as labelled precursors.

Material and Methods

Blood was obtained from eight normal individuals. The in vitro incubation mixtures were prepared by adding 2 ml of normal saline containing 100 μc of H^3-cytidine or 100 μc of H^3-leucine (the Radiochemical Centre, Amersham, England) to 8 ml of heparinized venous blood, so that the final concentration was 10 μc/ml.

The mixtures were incubated in siliconized test-tubes. After mixing, the tubes containing the mixtures with H^3-cytidine were left for 1 hour at 37 °C while the tubes with H^3-leucine were left at the same temperature for 2 hours. After incubation, during which erythrocytes settled, the supernatant was decanted and centrifuged at 1500 r.p.m. for 5 minutes. The buffy coats were separated and put in siliconized tubes, 2 to 3 drops of the supernatant plasma were added in order to resuspend the concentrated leukocytes. Leukocyte smears were made, dried rapidly and fixed in Carnoy' fluid. Autoradiographs were made in refrigerator at +2 °C in dry air with silica gel as desiccant the slides were generally exposed for 25 days, developped, washed, and stained with May-Grünwald Giemsa.

Grain counts were made visually with the aid of pair of 16× oculars and 100 oil immersion objective The percentages of labelled cells and the mean grain counts per cell for each group were obtained by counting H^3-labelled leukocytes in total of at least 2000 mononuclear cells. Cells with 4 or more grains over the nucleus were considered as labelled.

Results

Peripheral mononuclear blood cells have been grouped morphologically in 7 types, although assigning individual cells to certain types was occasionally difficult, and there seemed to be transition forms.

Type A Large round cells with central nucleus, with nucleolus and a very fine chromatin pattern of the kind usually associated with immaturity" and a narrow basophilic cytoplasm.

Type B Cells of smaller size than the above mentioned elements, with scanty basophilic cytoplasm and with a fine network of chromatin particles, largely mixed with areas of coarse chromatin masses.

Type C Small lymphocytes, without distinct cytoplasm or with a very thin layer and large coarse chromatin masses.

Type D Cells of a size larger than that of the group C, with cytoplasm always evident and chromatin less addensed than in small lymphocytes.

Type E. Large lymphocytes, with abundant cytoplasm and eccentrically located nucleus.

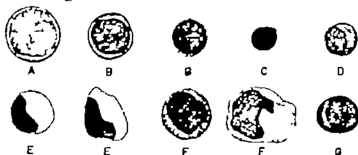


Fig 1 The drawings illustrate schematically the features of the cell types described in the text. Two most frequent aspects are given for the types B, E and F

Type F Cells of the monocytic type, usually with the well known morphological features of the mature cell of this type, occasionally with narrower basophilic cytoplasm and a non lobulated nucleus.

Type G Cells of the plasmocytic type. Many of them showed features of immaturity with a nucleus with a fine chromatin pattern.

The morphological features of the above described types of cells are schematized in fig 1 The percentages of the cells of the various types were different not only from one case to another but also in different slides of the same case. Evidently the method of concentrating leukocytes used does not permit to obtain the peripheral blood cells in a ratio representative of the actual "in vivo"

Table I

Percentage of labelling and mean grain count per cell. The figures indicate the arithmetic mean on eight cases.

Cell Type	Cytidine		Leucine		
	mean percentage of labelled cells	mean grain count/cell	mean percentage of labelled cells	mean grain count/cell	Cytidine Leucine ratio
A	100	83	100	15	5,5
B	100	65	100	17	3,8
C	91	37	78	12	3,0
D	92	39	91	32	1,2
E	78	42	97	49	0,8
F	100	51	100	63	0,7
G	100	115	100	95	1,2

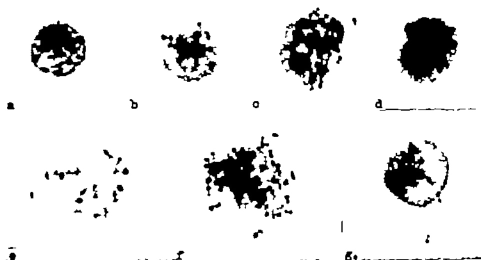


Fig. 2. Labelled cells after in vitro incubation of blood with H^3-cytidine or H^3-leucine. a) Labelled type C cell after incubation with H^3-cytidine. b) Labelled type C cell after incubation with H^3-leucine. c) Labelled type F cell after incubation with H^3-leucine. d) Labelled type E cell after incubation with H^3-cytidine. e) Labelled type F cell after incubation with H^3-cytidine. f) Labelled type F cell after incubation with H^3-leucine. g) Labelled type B cell after incubation with H^3-leucine.

ratio, so that the observed values are not reported. The mean percentage of labelling and the mean grain counts per cell are summarized in table I where the cytidine leucine ratio for each type of cell is also reported.

Discussion

DNA labelling is generally thought to occur only when cells synthesize DNA in preparation for mitosis. Since DNA synthesizing

cells in peripheral blood have always been found in a very small number in normal individuals (3) practically all cytidine uptake observed in the present study must be considered as RNA labelling However some questions arise in the interpretation of the labelling of RNA as well as of proteins after incubation with radioactive nucleosides and aminoacids. In fact, it cannot be automatically accepted as a measurement of nucleic acid and protein synthesis.

Exchange of the uridine-containing nucleotide has been demonstrated in vitro in isolated thymus nuclei without synthesis of RNA (1) the terminal cytidylyl-adenylyl complex of the soluble RNA might also be labelled by exchange (23) All RNA labelling observed in our experiments may not therefore be taken as evidence of synthesis. However many observations suggest that at least most of the RNA labelling with tritiated cytidine may be accepted as an indication of RNA synthesis (16) It must be added that uridine and cytidine give the same radioautographic pattern (22) while adenosine gives a pattern different from that of cytidine in the nucleus of the cells in tissue culture (10) Radioautographs obtained with H^3-cytidine cannot therefore be taken as representative of all types of RNA.

The aminoacids incorporation, although it can also represent aminoacid activation or exchange, is now generally considered as an indication that a segment of a peptide chain is synthetized (19) CARNEIRO AND LEBLOND (4) and SCHULTZE et al. (21) found the same radioautographic pattern of protein synthesis no matter whether leucine, lysine, methionine or glycine were used there is further evidence (12) that protein labelling with l leucine can be regarded as representative of protein synthesis.

Our results clearly show that RNA and protein synthesis take place in a great number of mononuclear peripheral cells This observation is in agreement with the well known fact that both RNA and proteins are synthetized continuously in the majority of cells (12) As far as peripheral leukocytes are concerned, a positive correlation between RNA synthesis and protein synthesis can by no means be observed in fact, in some types of cells to a high rate of cytidine incorporation corresponds a low rate of aminoacids incorporation.

The chief importance must be attributed to the behaviour of the RNA synthesis/protein synthesis ratio in circulating cells of the

"lymphatic" types. The variability of the ratio in these cells may obviously depend on the fact that lymphocytes in blood are a heterogeneous population (24) derived from several sources other than thoracic duct lymph, and apparently consisting of a number of cell groups which differ in their potentialities even though they are morphologically very similar. Thus, wide differences in metabolic activity are observed among these peripheral elements. As a matter of fact, in the cells of the group E the protein synthesis appears to be the highest among the cells of the "lymphatic" types, so that the RNA synthesis/protein synthesis ratio amount roughly to one, resembling that of the monocytes. On the other hand in the cells of the group A, only a very small uptake of H^3-leucine corresponds to a heavy labelling with H^3-cytidine, so that the RNA synthesis/protein synthesis ratio is the highest among all the circulating mononuclear cells, amounting roughly to five. Since synthesis of (specific) proteins is one of the chief metabolic features related to cell differentiation, these cells, showing the most marked morphological characteristics of immaturity may be regarded from the metabolic as well as from the morphological point of view as the least differentiated circulating elements.

Furthermore, it must be emphasized that a high RNA synthesis/protein synthesis ratio is found also in the majority of small lymphocytes. As a matter of fact, many cells of this type show a high H^3-cytidine uptake, a result in disagreement with the view that the small lymphocyte is a final stage like the granulocyte. As can be easily observed in the table I the RNA synthesis/protein synthesis ratio in these cells is quantitatively similar to that of the immature cells of the types A and B Since we are now aware that small lymphocytes can change rapidly into dividing cells with new morphological and functional characteristics (5 13) and that they have to be considered as immunologically competent cells (9) they cannot be regarded as mature final cells. It seems therefore possible to think that analogous metabolic mechanisms are active in the cells of the types A and C.

The highest rate of protein synthesis was observed in the cells of the plasmocytic type, an observation quite in agreement with the well known strong development in the cytoplasm of these cells, of the ergastoplasm and of the Golgi body (2) two cellular organelles considered to take a main part in the elaboration and excretion of proteins.

Beside the plasmocytes, a high protein synthesis was observed, in lymphocytes of the type E and in monocytes. As far as these cells are concerned, it must be emphasized that the synthetic activity of these elements appears to be quite different from that of the cells of the type A, which we consider as the least differentiated circulating cells. Our observation seems to be in agreement with the view that the monocytes cannot be regarded as pluripotential cell (6).

As far the cytological significance of the dissociation between RNA and protein synthesis some further remarks must be added. It is a well known fact that the nucleus contains several RNA fractions with a very different turnover rate some of these fractions have a very high turnover rate. Thus a large proportion of nuclear RNA synthesis takes place without a corresponding increase in total RNA content (14) Autoradiographic and chemical studies have shown that the rapid nuclear RNA turnover is mainly due to continuous turnover of nucleolar RNA, which takes place at different rates according to the degree of cell differentiation (20) On the other hand there is no turnover of proteins in the fully grown nucleolus (20) so that the nucleolar synthesis of RNA is much greater than that of proteins (12)

It may therefore be assumed that in resting blood cells, in which a high H^3-cytidine and a low H-leucine uptake can be observed, the metabolic processes related to the nucleolus and to the associated structures are the main nuclear activity This hypothesis seems plausible not only for the well nucleolated cells of the group A, but also for the small lymphocytes, in which the electrone microscope generally reveals a nucleolus surrounded by chromatin.

Summary

Peripheral leukocytes were incubated with either H^3-cytidine or H^3-leucine, and radioautographs wer prepared. Mononuclear cells were subdivided into seven groups, according to their morphology and the mean percentage of labelling and the mean grain count for each group was obtained. No direct relationship was observed between H^3-cytidine and H^3-leucine uptake. While in some types of cells, such as the monocytes, the ratio amounted roughly to one. In other types of cells, such as the circulating cells of "blastic" type, the ratio was much higher I is emphasized that in large lymphocytes the cytidine/leucine ratio resembles that observed in monocytes, while in small lymphocytes the ratio is similar to that of the cells of "blastic" type. I is assumed that in resting blood cells with high H^3-cytidine and low H^3-leucine uptake, the metabolic processes of the nucleolus and the associated structures represent the main nuclear activity

Résumé

Les auteurs incubent des leucocytes du sang périphérique soit dans la cytidine-H^3 soit dans la leucine-H^3 puis procèdent à une étude radioautographique. Les cellules mononucléées sont reparties en 7 groupes d'après leur morphologie. Pour chaque groupe, l'intensité d[illegible] marquage (en pourcent) et le nombre moyen de granulations sont notés. On ne peut trouver une relation directe entre la résorption de la cytidine-H^3 et celle de la leucin-H^3 Alors que la valeur de cette relation atteint presque un chez quelques *formes cellulaires, comme les monocytes, elle reste sensiblement plus élevée chez d'autres* types de cellules, comme les cellules d[illegible] type [illegible] blastes [illegible] en circulation. Les auteurs font remarquer que la relation de la cytidine à la leucine des grands lymphocytes ressemble à celle des monocytes, alors que la relation des petits lymphocytes ressemble à celle du type [illegible] blastes» On peut admettre que le métabolisme du nucléole et la structure cor respondante expriment l'activité moyenne du noyau des cellules sanguines en repos [illegible]vec une résorption forte de cytidine-H[illegible] et faible de leucine H^3

Zusammenfassung

Periphere Leukozyten wurden entweder mit H^3-Cytidin oder mit H^3-Leucin inkubiert und radioautographisch untersucht. Die mononukleären Zellen wurden auf Grund ihrer Morphologie in 7 Gruppen eingeteilt. Für jede Gruppe wurden die prozentuale Markierung und der mittlere Gehalt an Granula bestimmt. Es fand sich keine direkt[illegible] Beziehung zwischen den Aufnahmen von H^3-Cytidin und von H^3 Leucin. Während dieses Verhältnis bei einigen Zellformen, wie den Monozyten, nahezu eins betrug, war es bei anderen Zelltypen, wie den zirkulierenden Zellen [illegible]om «Blastem»-Typ, wesentlich höher Es wird darauf hingewiesen, daß bei den großen Lymphozyten das Ver hältnis von Cytidin zu Leucin demjenigen der Monozyten gleicht, während es bei den kleinen Lymphozyten ähnlich ist wie in den Zellen vom «Blastem»-Typ. Es wird angenommen, daß in ruhenden Blutzellen mit hoher H^3-Cytidin- und niedriger H^3-Leucin-Aufnahme die Stoffwechselprozesse des Nucleolus und der damit verbundenen Struk turen die mittlere Kern-Aktivität wiedergeben.

References

1 ALLFREY V AND MIRSKY A. E. Contrasts in the metabolic stability of different nucleotides in the ribonucleic acids of isolated nuclei. Proc. Nat. Acad. Sci., Wash. *45*. 1525–1344 (1959)

2. BESSIS, M. C. Ultrastructure of lymphoid and plasma cells in relation to globulin and antibody formation. Lab. Invest. *10*: 1040–1067 (1961)

3 BOND, V P FLIEDNER, T M. CRONKITE, E. P RUBINI, J B. BRECHER, G. AND SCHORK, P K. Proliferative potentials of bone marrow and blood cells studied by in vitro uptake of H^3-thymidine. Acta haemat. *21* 1 15 (1959)

4. CARNEIRO J AND LEBLOND, C. P Continuous protein synthesis in nuclei shown by radioautography with H^3-labelled aminoacids. Science *129*· 391 393 (1959)

5. CASTALDI, K. The human lymphocyte its possible pluripotential quality Lancet *i*. 829–830 (1962)

6. COLE, L. J General discussion on transplantation of bone marrow J cell. comp. Physiol. *50*: 163–171 (1957)

7 COLE, L. J AND GAR[illegible]ER, R. M. Homograft reactive large mononuclear leukocytes in peripheral blood and peritoneal exudates. Amer J Physiol. *200*· 147–151 (1961)

8. GOODMAN J W AND HODGSON, G S. Evidence for stem cells in [illegible]he peripheral blood of mice. Blood *19* 702 714 (1962)

9. Gowans, J. L., McGregor, D. D.; Cowen, D. M. and Ford, C. E. Initiation of immune responses by small lymphocytes. Nature 196: 651–655 (1962).
10. Harris, H.: Turnover of nuclear and cytoplasmic ribonucleic acid in two types of animal cells, with some further observations on the nucleolus. Biochem. J. 73: 362–369 (1959).
11. Lajtha, L. G.; Oliver, R. and Gurney, C. W. Kinetic model of bone marrow stem-cell population. Brit. J. Haemat. 8: 442–460 (1962).
12. Leblond, C. P. and Amano, M. Synthetic activity in the nucleolus as compared to that in the rest of the cell. J. Histochem. Cytochem. 10: 162–174 (1962).
13. McKinney, A. A., jr.; Stohlman, F. jr. and Brecher, G. The kinetics of cell proliferation in cultures of human peripheral blood. Blood 19: 349–358 (1962).
14. McMaster-Kaye, R. The metabolism of nuclear ribonucleic acid in salivary glands of Drosophila repleta. J. Histochem. Cytochem. 10: 154–161 (1962).
15. Nowell, P. C. Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. Cancer Res. 20: 462–466 (1960).
16. Perry, R. P.; Errera, M.; Hell, A. and Durwald, H. Kinetics of nucleoside incorporation into nuclear and cytoplasmic RNA. J. biophys. biochem. Cytol. 11: 1–13 (1961).
17. Pileri, A.; Marasco, G. and Gavosto, F. Interrelationship between RNA and protein metabolism in human normal bone marrow cells. Exp. Cell Res. 20: 645–646 (1960).
18. Popp, R. A.: Erythrocyte repopulation in X-irradiated recipients of nucleated peripheral blood cells of normal mice. Proc. Soc. exp. Biol., N. Y. 104: 722–724 (1960).
19. Raacke, I. D. The synthesis of proteins; in Greenberg's Metabolic Patways, vol. II, pp. 263–388 (Academic Press, New York/London 1961).
20. Sirlin, J. L. The nucleolus. Progr. Biophys. biophys. Chem. 12: 25–66 (1962).
21. Schultze, B.; Oehlert, W. and Maurer, W. Vergleichende autoradiographische Untersuchung mit H³,C¹⁴-S³⁵-markierten Aminosäuren zur Größe des Eiweißstoffwechsels einzelner Gewebe und Zellarten bei Maus, Ratte und Kaninchen. Beitr. path. Anat. 122: 406–431 (1960).
22. Schultze, B.; Oehlert, W. und Maurer, W. Über eine allgemeine Beziehung zwischen der Umsatzrate der Ribonucleinsäure und des Eiweißes im Organismus von Maus und Ratte. Biochim. biophys. Acta 49: 35–46 (1961).
23. Vincent, W. S. and Baltus, E. The ribonucleic acids of nucleoli; in Mitchell The Cell Nucleus, pp. 18–23 (Butterworth, London 1960).
24. Yoffey, J. M. Quantitative Cellular Haematology (Thomas Springfield, Ill. 1960).

Authors' address: Drs. U. Torelli, G. Grossi, T. Artusi and G. Emilia, Istituto di Patologia Medica, Policlinico, *Modena* (Italy).

Table II

The effect of radiotherapy upon the consumption test.

Diagnosis	Area of treatment	Field size	Tumor dose rad	Consumption test before/after treatment: Thrombocytes		Leucocytes		W.B.C. before/after treatment
Carcinoma of maxillary sinus	Max. sinus	8×8 ant. + lat.	5000	ø	3	ø	ø	6750/595
Hodgkin's disease	Skull	10×10 4 fields	2500	ø	2	ø	ø	7000/650
Malignant melanoma	Skull	10×10 4 fields	5000	2	2	1	1	8000/800
Lymphoepithelioma of nasopharynx	Mastoid	10×10	2000	4	4	ø	3	4000/400
Lymphosarcoma	Supraclavicular	10×10	2000	ø	4	3	3	6500/650
Carcinoma of thyroid	Cervical + supraclavicular	10×17 2 fields	5000	ø	4	ø	1	7000/420
Hodgkin disease	Cervical	10×15 2 fields	2000	2	4	2	2	9300/650
Hodgkin disease	Mediastinum	10×15 2 fields	2000	4	4	3	4	5900/640
Hodgkin's disease	Thorax-sinist.	10×15 2 fields	2000	1	ø	3	ø	8100/580
Bronchial carcinoma	Thorax-dext.	10×15 3 fields	3000	ø	3	ø	ø	7000/450
Bronchial carcinoma	Thorax-sinist.	18×16 2 fields	3000	4	ø	3	ø	11100/105
Esophageal carcinoma	Lower esophagus	10×17 2 fields	4000	2	4	ø	3	7800/580
Carcinoma of stomach	Upper abdomen	15×15 4 fields	2500	3	ø	ø	ø	4200/200
Carcinoma of sigmoid	Lower abdomen	20×20 4 fields	3000	ø	4	ø	2	5500/450
…rcinoma of ovary	Upper abdomen	10×20 2 fields	2000	ø	ø	ø	ø	7850/450
… of ovary	Upper abdomen	20×20 4 fields	3000	1	1	ø	ø	6800/580
…oma of ovary	Lower abdomen	10×20 4 fields	2500	4	4	2	3	8000/420
Carcinoma of breast	Liver	10×15 2 fields	1000	4	3	ø	ø	7900/570
Hodgkin disease	Upper abdomen	10×15 2 fields	2000	4	4	4	4	11000/560
Lymphosarcoma	Upper abdomen	20×20	800	3	1	ø	ø	4000/200
Lymphosarcoma	All peripheral lymph nodes	10×10 8 fields	2000 each field	4	2	4	2	7000/490
Chronic myeloid leukemia	Spleen	10×15	850r*	ø	4	ø	4	23000/15
Chronic myeloid leukemia	Spleen	10×15	1050r*	ø	4	ø	4	21000/10
Chronic myeloid leukemia	Spleen	10×15	1125r*	3	4	2	ø	20000/72
Chronic myeloid leukemia	Spleen	10×15	800r*	2	3	ø	4	100000/48
Chronic myeloid leukemia	Spleen	10×15	900r*	ø	1	ø	±	102800/270
Lymphosarcoma	Spleen	10×15	250r*	ø	3	ø	1	5800/50
Chronic lymphatic leukemia	Spleen	10×15	1450r*	ø	ø	ø	ø	190000/54

Air dose

Table III

Changes in the consumption test after the end of treatment.

Diagnosis	Consumption test at end of treatment		Time since end of treatment	Follow up consumption test	
	Thrombocytes	Leucocytes		Thrombocytes	Leucocytes
Chronic myeloid leukemia	4	4	5 weeks	4	1
			4 months	1	1
Chronic myeloid leukemia	4	Ø	3 weeks	2	Ø
			1 week	1	Ø
Chronic myeloid leukemia	4	4	9 months	2	Ø
Chronic myeloid leukemia	3	4	4 months	3	2
Hodgkin disease	4	2	5 weeks	2	2
Hodgkin disease	4	4	9 months	1	
Lymphosarcoma	4	3	9 months	1	1
Lymphosarcoma	3	Ø	5 weeks	Ø	Ø
Lymphosarcoma	2	2	3 months	1	1
Reticulum cell sarcoma	4		3 weeks	3	
Polycythemia		2	5 weeks	Ø	Ø
Carcinoma of breast	4	2	10 days	2	Ø
Carcinoma of breast	3	4	5 weeks	2	
Carcinoma of breast	4	3	4 months	4	2

test showed no change or its degree of positivity became reduced. Eight of these received radiotherapy to the upper abdomen and left hemithorax. In no patient who received treatment to these areas did the consumption test become stronger a phenomenon we find difficult to explain. Animal experiments are being undertaken to try and throw light on this problem. Of the three other patients in whom no positivity occurred one with malignant melanoma received radiotherapy to the skull, an area which, when irradiated nearly always produces very slight or no hematological changes. The patient with lymphatic leukemia received irradiation to the spleen. In this case, the number of lymphocytes was reduced, which did not occur with the other patients in whom granulocytes were mostly affected. This may account for the lack of change in the test. The patient with lymphosarcoma had enlargement of all the peripheral lymph nodes, and it is possible that this accounted for the strong positive test before treatment. After successful treatment the test became less positive, and the remaining positivity might be due to the X ray treatment and not to the disease. The area of treatment, size of field and the dose, seem to have an influence upon the result of the test.

The DACT is a more sensitive test than the usual tests for the detection of antibodies in the serum and this accounts for the high percentage of positive results. Whether the coating globulin causing the positive test is an antibody or a globulin attached to a damaged cell is not yet settled (3). The finding of positive or more strongly positive DACT after radiological or chemotherapeutical treatment supports the second possibility. The positive test in a patient who has not had any previous treatment may be due to some globulins produced by the tumour itself. The mere coating of the cells with globulin may interfere with cell metabolism and shorten its life.

Summary

Interference in white blood cell and thrombocytes metabolism was found in some of the patients with malignant disease who had not been treated previously. These changes were similar to those observed after radiotherapy and chemotherapy but in lesser degree. The direct antiglobulin consumption test (DACT) became positive or more positive if it was already so, in all patients getting chemotherapy. Local radiotherapy compared to chemotherapy produced similar but less marked changes in the test. The DACT was repeated after intervals without further treatment and the degree of positivity decreased, or the test became negative.

Résumé

Les auteurs démontrent la présence de troubles métaboliques de leucocytes et des thrombocytes chez des malades atteints de tumeurs malignes, qui [illegible] ont pas été traités auparavant. Les modifications du métabolisme ressemblent à celles observées après radio- ou chimiothérapie, sans en montrer l'ampleur. Le test direct de consomption de l'antiglobuline (DACT) était positif chez tous les malades soumis à la chimiothérapie, ou, [illegible]'il était déjà positif [illegible]vant le traitement, devenait plus fortement positif. En comparaison [illegible]vec la chimiothérapie les mêmes modifications du [illegible] est survaient une radiothérapie locale, mais elles étaient moins fortes après une interruption du traitement le DACT devenait alors plus faible ou négatif.

Zusammenfassung

Bei einigen zuvor nicht behandelten Patienten mit malignen Tumoren wurden Stoffwechselstörungen von Leukozyten und Thrombozyten nachgewiesen. Diese Veränderungen waren ähnlich, [illegible]ber weniger ausgesprochen wie diejenigen nach Radiotherapie und Chemotherapie. Der direkte Antiglobulin-Consumptionstest (DACT) war da bei allen einer Chemotherapie unterworfenen Patienten positiv oder wenn er schon vorher positiv war wurde er stärker positiv. Im Vergleich zur Chemotherapie führte eine lokale Radiotherapie zu ähnlichen, aber weniger ausgesprochenen Veränderungen des Testes. Der DACT wurde nach einem behandlungsfreien Intervall schwächer oder negativ

References

1. Edelman, A.: New evidence for toxic substance elaborated after irradiation. Fed. Proc. *14*: 42 (1955).
2. Osgood, E. E.: Is the action of roentgen rays direct or indirect? Amer. J. Roentgenol. *48*: 214 (1942).
3. Nelken, D.: Nature of the coating globulin causing a positive antiglobulin consumption test. Nature *191*: 1110 (1961).
4. Nelken, D.; Gurevitch, J. and Gilboa-Garber, N.: Direct anti-globulin consumption test for detection of immune antibodies. Lancet *i*: 742 (1961).

Authors' addresses: Dr. E. Robinson, Dept. of Oncology; Dr. D. Nelken, Dept. of Clinical Microbiology, Hadassah Univ. Hospital, P. O. Box 499, Jerusalem (Israel).

Acta haemat. 30: 144–152 (1963).

From the Department of Internal Medicine, Allmänna Sjukhus, Malmö (Prof. JAN WALDENSTRÖM)

The Significance of the "Protein Error" of Indicators in the Diagnosis of Bence-Jones Proteinuria

By J. K. SMITH

Since the studies of NIETZKI AND BURCKHARDT (15) and SORENSEN (19) a phenomenon known as the "protein error" of indicators has been known to occur with certain pH indicators in the presence of protein. It consists of a displacement in the transformation range of the indicator which is independent of changes in the pH and has been ascribed to the formation of a salt like adsorption compound between the protein and the indicator (15). The color shift may be towards the alkaline or the acid side. The reaction is not entirely specific for native proteins, since some colorimetric discrepancy has been noted to occur in solutions containing high concentrations of products of proteolysis (4) and since a significant transformation may occur in the presence of neutral salts (generally alkaloids of high molecular weight) (4, 5).

The present investigation was prompted by the observation that large quantities of *Bence-Jones* proteins frequently failed to induce any significant alteration in the transformation range of a citrate buffered (pH 3.5) tetrabromphenolsulfonphthalein impregnated filter paper strip* although significantly positive results were obtained when the urines were tested with several other laboratory procedures used routinely in the detection of proteinuria.

A comparison is made between the relative abilities of four frequently used laboratory methods to detect the presence of M components ("abnormal" globulins) in urines from 19 patients with multiple myeloma or macroglobulinaemia. Discrepancies between the behavior of the colorimetric filter strip in the presence of albumin

* Albustix, Ames Co.

Table I

Patient	Diagnosis	Albustix	Heller	SSA	Purdy	[illegible] Test	Biuret (g%)	[illegible]
V A.	Myeloma	0	2 4	4	4	+	2.94	γ_1
B. W	Myeloma	30–100 mg	4	4	4	+	1.41	γ
E. H.	Myeloma, paramyloid	0	4	4	4	+	1.03	γ_1
Mg. O.	Myeloma	0	4	3	2	+	0.890	α_2
A. A.	Myeloma	30 mg	3	1	1	+	0.688	γ_1
T J	Myeloma	30 mg	3	3	3	+	0.441	γ
Ado.	Myeloma	0	2	3	3	+	0.264	γ_1
K. O	Myeloma	30 mg	3	2	3	+	0.240	β mod. albumin
G. R.	Myeloma	30 mg	1	2	0	0	0.190	γ trace albumin
L. J.	Myeloma	30 mg	1	1	1	0	0.132	β trace albumin
E. B.	Macroglob.	0	2	1	2	0	0.104	β
M. G.	Myeloma	0	1	tr	1	0	0.092	γ_1 trace albumin
L. L.	Myeloma	30 mg	1+	2	2	0	0.084	β mod. albumin
M. O.	Macroglob.	0	tr	tr	tr	0	0.082	γ γ_1 trace albumin
K. N.	Myeloma	30 mg	1	2	1	0	0.068	γ_1 (β) mod. albumin
San.	Myeloma	0	tr	0	0	0	0.044	α_2 trace albumin
B. C.	Myeloma	0	tr	0	0	0	0.038	α_2 trace albumin
G. M.	Myeloma	0	tr	0	0	0	0.033	β γ_1 trace albumin
F P	Myeloma	0	tr.	tr	0	0	0.024	α_2 trace albumin

Same results at pH 5.0 and pH 8.0.

** Criteria for positive reactions: trace = less than 2 mm precipitate, 1 = 2 3 mm, 2 = 4–5 mm, 3 = 6 mm, 4 = more than 6 mm.

Criteria for positive reactions trace = faint turbidity 1 = definite turbidity no granulation, 2 = granulation, no flocculation, 3 = marked flocculation, 4 = precipitation.

† Because of the 1 1 dilution of the urines with the phosphate buffer in this test, the protein concentrations are one half the values listed under Biuret.

and in the presence of gamma globulin and 12 isolated urinary M components are noted, and the theoretical significance briefly discussed. The combined findings of a negative or trace positive colorimetric strip test and a strongly or moderately positive *Heller's* test, *Purdy's* heat test, or sulfosalicylic acid test are shown to strongly favor the diagnosis of gamma related proteins in the urine*

Materials and Methods

Urine collection. Freshly voided urine samples from patients with multiple myeloma or macroglobulinaemia were filtered and tested immediately. Urine samples not being used were acidified and stored in polyethylene containers at 4 C. Toluene was not used as preservative since this may result in false negative reactions with the colorimetric strip test due to the development of water repellence by the filter paper (7).

* Gamma-related globulins include the 7S and 19S gamma globulins, myeloma serum globulins, pathological macroglobulins, and Bence-Jones proteins (20).

Routine studies: Each urine sample was tested with Albustix at the original pH, and at pH 5.0 and 8.0 following adjustment with HCl or NaOH, respectively. The strips were read in double blind study the mechanics of the testing being similar to those recommended by FRASER (7). Results were recorded in terms of 0, 30, 100, 300 or 1000+ mg. of protein using the color scale provided with the indicator strips. pH determinations were performed with a Beckman glass electrode meter (Model H 2) with an accuracy of ± 0.1 units.

Other routine studies for proteinuria included the sulfosalicylic acid test (10), *Purdy's* heat test (21) and *Heller's* test (10). The criteria for positive reactions in these tests are listed in table I.

2.0 ml urine samples were buffered with 2.0 ml of 0.01 molar Na_2HPO – 0.07 molar NaH_2PO solution of pH 6.0, ionic strength 0.1 and tested for the presence of *Bence-Jones* proteins by heating slowly to 60 C. Proteins which precipitated between 45 and 60 C, partly or completely redissolved when heated to 100 C, and reprecipitated when cooled to room temperature, were considered to be *Bence-Jones* proteins.

Total proteins were determined by the Biuret method (8) following the precipitation of 5 ml urine samples with 10% trichloroacetic acid as suggested by FORTAN et al. (6). Supernatants were also checked for protein content since trichloroacetic acid may fail to precipitate some of the lower molecular weight proteins. Any remaining fractions were precipitated with 60% sodium sulfate and included in the protein determination.

Electrophoresis: Urine samples were concentrated to protein content of 0.5 gm/100 ml or more by dialysis against 15% aqueous carboxymethylcellulose at 4 C. 0.2 ml samples were then subjected to paper electrophoresis for 16 hours t 140 volts using diethyl barbiturate buffer of pH 8.6, 0.1 ionic strength.

Separation of M-components: 12 urinary concentrates were dialyzed for 8 hours against two changes of the aforementioned phosphate buffer and precipitated by the addition of solid ammonium sulfate to 60% saturation at 22 C. The precipitates were dissolved in 0.9% NaCl and dialyzed for 12 hours against three changes of distilled water at 4 C to assure the complete removal of ammonium sulfate. The proteins (many of which were water insoluble) were subsequently equilibrated by dialysis with the phosphate buffer and adjusted to protein concentrations of 16 to 1000 mg/100 ml. Electrophoretic analyses were repeated, and the proteins found to be free of albumin contaminants. The protein solutions were then retested with Albustix.

Albumin and gamma globulin: Similar concentrations of phosphate buffered purified human serum albumin and purified serum gamma globulin (Kabi, Stockholm, Sweden) were prepared and tested with the indicator strips. Immunoelectrophoresis of the latter protein, using the method of SCHEIDEGGER (17) as modified by HERMANS (11) with antihuman antisera (Behring-Werke) as the antibody source, revealed it to be a homogeneous gamma globulin with minor alpha₂ macroglobulin contaminant, and mobility on paper electrophoresis of $gamma_1$, $gamma_2$ and $gamma_3$. Analysis of the albumin by the supplier revealed minor contaminating fractions of transferrin (0.8%) and alpha globulin (1.0%).

Results

Urinary protein concentrations as determined by the Biuret method varied from 24 to 2,940 mg/100 ml in the presenting unconcentrated samples. The mobilities of the M-components on paper electrophoresis ranged from $alpha_2$ to $gamma_3$, with a predominance in the $gamma_1$ range. Of the 19 urines studied, 3 (15.8%) (M. O., K. N., and G. M.) had more than one M-com

ponent, and 11 (57.9 %) had detectable amounts of albumin on paper electrophoresis, although in every instance the M-component predominated. The heat flocculation test for the detection of *Bence-Jones* proteins was positive in 8 of 19 urines (42.1 %) and was negative in all samples diluted with buffer to concentrations of 95 mg/100 ml of protein or less regardless of the mobility of the components* (table I)

Detection of urinary M-components by routine laboratory procedures
All of the 19 unconcentrated urine samples reacted positively with *Heller's* nitric acid test. The quantitative correlation with this method was not always reliable, however as may be seen in table I

The sulfosalicylic acid test was positive in 16 of the 19 urines (84.2 %) and was negative only in those samples containing less than 50 mg/100 ml of protein. The method, with certain exceptions (urines from A. A. L. L., and K. N) gave a fairly reliable quantitative correlation.

Purdy's heat test was positive in 14 of the 19 urines (73 7%) and was also negative in urine samples containing less than 50 mg/100 ml of protein. One urine sample (G R.) containing 190 mg/100 ml of protein, however did fail to precipitate upon heating to 100 C. The quantitative correlation was similar to that obtained with the sulfosalicylic acid test.

The citrate-buffered tetrabromphenolsulfonphthalein impregnated filter strip test for proteinuria was minimally positive in 8 of 19 urines (42.1 %) of which all but three contained detectable amounts of albumin on electrophoresis. In only one instance (B.W) was the reaction of the colorimetric test read as being greater than 30 mg (i. e. 30–100 mg) despite the fact that the urinary protein concentrations ranged as high as 1410 mg/100 ml, and were above 130 mg/100 ml in 6 of the 8 reacting samples (table I) Both of the urines reacting with less than 100 mg/100 ml of protein (L. L. and K. N) had moderate to heavy albumin components.

Eleven urines failed to induce any change in the colorimetric strip of these, 7 (63.6%) had protein concentrations of greater than 80 mg/100 ml, and 4 (36.4 %) had protein concentrations greater than 250 mg/100 ml The most striking examples of the failure of some components to induce any transformation in the in

* In separate study it was noted that the precipitate resulting from the heating of known Bence-Jones proteins in concentrations of less than 50–60 mg/100 ml was generally so minimal as to be readily overlooked without spectrophotometric aids.

Table II

Reaction of albustix to phosphate buffered albumin, γ-globulin, and 12 isolated urinary M-components.

Patient	Mobility of M-Component	Concentration of Protein in g/100 ml 1.0	0.5	0.25	0.125	0.063	0.032	0.016
Mg. O.	α_2	0	0	0	0	0	0	0
K. O.	β	100	30–100	30+	30—	0	0	0
L. L.	β	–	100	100—	30–100	30+	30—	0
B. C.	β	30	30—	0	0	0	0	0
K. N	$\gamma_1(\beta)$	30+	30	30—	0	0	0	0
V Å.	γ_1	0	0	0	0	0	0	0
E. H.	γ	0	0	0	0	0	0	0
A. A.	γ_1	30	30—	0	0	0	0	0
T J	γ_1	100	30+	30	30—	0	0	0
M. O.	γ (γ_1)	–	30+	30	30—	0	0	0
Ado.	γ	30+	30—	0	0	0	0	0
B. W	γ	30—	0	0	0	0	0	0
	Albumin	1000+	300+	100+	100	30–100	30	0
	γ-Globulin	30+	30	30—	0	0	0	0

dicator strip were the urines from V Å. and E. H. in which the protein concentrations were 2940 and 1030 mg/100 ml, respectively Both of these components were of gamma$_1$ mobility No statistically significant correlation could be found between the mobilities of the components on paper electrophoresis and their abilities to affect the dissociation of the tetrabromphenolsulfonphthalein impregnated filter strip however

Comparison of the behavior of tetrabromphenolsulfonphthalein in the presence of albumin, gamma globulin, and isolated M-components. The responses of the indicator strips to purified human serum albumin and gamma globulin and to 12 phosphate buffered, ammonium sulfate fractionated M-components of concentrations ranging from 16 to 1000 mg/100 ml are listed in table II. Although the M-components varied in their abilities to affect the dissociation of tetrabromphenolsulfonphthalein, their behavior in general was similar to that of purified gamma globulin in no instance did they result in an alkaline shift of the indicator equal to that caused by an identical concentration of albumin. Five of the components (41 7%) failed to induce any change below concentrations of 250 mg /100 ml. of protein, and three (25 /) had no demonstrable effect on the indicator dissociation. Only one of the 12 components (8.3%) could be expected to induce a colorimetric response in concentrations below 125 mg/100 ml Again no correlation could be drawn between the mobilities of the components on paper electrophoresis and

their abilities to affect the dissociation of the indicator in concentrations and pH conditions present in the filter strip. All but one (K. N.) of the isolated components demonstrated heat-solubility characteristics typical of *Bence-Jones* proteins when present in a phosphate buffer pH 6.0 in concentrations of 250 mg/100 ml.

Discussion

The diagnosis of *Bence-Jones* proteinuria has been a perplexing problem for the physician since the original description of one member of this heterogeneous group of proteins by BENCE JONES in 1848 (2). Although electrophoretic analysis of concentrated urine samples in all patients with proteinuria undoubtedly offers the most reliable means of detecting gamma related globulins in the urine, the method is impractical as a routine screening procedure. In addition, the final diagnosis of *Bence-Jones* proteinuria has traditionally depended on the demonstration of heat-solubility characteristics typical of these proteins, although, more recently methods such as ultracentrifugation and the immuno-diffusion techniques have added greatly to the diagnostic armament.

The present study confirms the reliability of the sulfosalicylic acid test and *Purdy's* heat test to detect the presence of urinary M components in concentrations greater than 50 mg/100 ml although, of course, these methods are nonspecific, and yield no information as to the nature of the proteins precipitated. In addition, *Heller's* nitric acid test was found to detect the presence of these proteins in all of the 19 samples, and in concentrations as low as 24 mg/100 ml, a factor which favors its use as a screening procedure for proteinuria. Heat-solubility characteristics typical of *Bence-Jones* proteins were noted in only 42.1% of the urines, a finding in keeping with the previously recorded experiences in the literature.

The marked discrepancies between the behavior of tetrabromphenolsulfonphthalein in the presence of albumin and in the presence of gamma globulin and 12 isolated urinary M-components are of both practical and theoretical significance. Although the value of the colorimetric strip test in the diagnosis of albuminuria has been established by numerous investigators (1 5 7 14) and confirmed in this investigation, it appears unlikely that a significant number of gamma-related urinary components will be detectable by this method in concentrations below 125 to 250 mg/100 ml

even then, the colorimetric response may be much less than one would expect with equivalent amounts of albumin, or it may be absent altogether It is suggested, therefore, that the combined findings of negative or trace positive colorimetric strip test and a strongly or moderately positive *Heller*'s test, *Purdy*'s heat test, or sulfosalicylic acid test may be considered to strongly favor the presence of gamma related proteins in the urine. Because of the not uncommon occurrence of albumin in urines with *Bence Jones* proteins (18) however a strongly positive colorimetric reaction does not rule out the presence of gamma related components in the urine.

The physicochemical significance of the variation in the abilities of albumin, gamma globulin and *Bence-Jones* proteins to affect the dissociation of tetrabromphenolsulfonphthalein is presently under investigation. Preliminary results in this laboratory suggest that the reaction (at pH's *below* the isoelectric point of the protein being tested) is due to binding of the anionic sulfonic acid group of the indicator to cationic sites on the proteins, of which the free amino acid groups appear to be most important. These findings are in keeping with the published work of JENKS et al. (12) in which they studied the abilities of electrophoretically separated protein fractions to bind tetrabromphenolsulfonphthalein and found that the binding capacities of human serum albumin and bovine albumin were considerably greater than those of the alpha, beta and gamma globulins, and that this binding could be decreased 58% by deamination with nitrous acid. In addition, these authors noted a positive correlation between the dye combining capacities and the number of free amino groups in serum albumin, bovine serum albumin, and gamma globulin. Furthermore, it has been demonstrated by GRASSMANN AND HANNIG (9) that the basic protein clupein has a greater dye uptake than albumin, whereas the acidic protein accounts for little dye uptake.

A study of 14 purified *Bence-Jones* proteins by PUTNAM AND MIYAKE is of particular interest (16) These investigators demonstrated that of eight proteins immunologically classified as belonging to antigenic type A of KORNGOLD (13) five lacked detectable N terminal amino acids, and all eight lacked N-terminal aspartic acid. Conversely those proteins that were immunologically classified as belonging to *Korngold*'s antigenic type B all contained N terminal aspartic acid, and had a greater overall abundance of N-terminal amino acids.

Although N terminal amino acid analyses and immunological classifications were not done in the present study it is suggested that the relative inability of the gamma related proteins to affect the dissociation of tetrabromphenolsulfonphthalein may be related to a deficiency in N terminal amino acids it is also possible that those *Bence-Jones* proteins which failed completely to react with the indicator may be of antigenic type A.

Acknowledgement: The author is grateful to J. V. Walcerman of the Ames Company for supplying useful information regarding their product.

Summary

A comparison is made between the relative abilities of four frequently used laboratory methods to detect the presence of "abnormal" globulins in the urines of 19 patients with multiple myeloma or macroglobulinaemia. Discrepancies between the abilities of albumin, gamma globulin and 12 urinary M-components to displace the transformation range of an indicator strip test for proteinuria are noted, and the theoretical significance discussed. The combined findings of negative or trace positive colorimetric strip test and strongly or moderately positive Heller test, Purdy heat test, or sulfosalicylic acid test are shown to strongly favor the presence of gamma-related proteins in the urine.

Résumé

L'auteur compare 4 méthodes de laboratoire courantes pour la recherche de globulines «anormales» dans l'urine de 19 malades atteints d'un plasmocytome ou d'une macroglobulinémie. On note des différences de la réaction de l'albumine, de la gamma-globuline et de 12 composés M de l'urine à l'aide d'un tampon indicateur servant à la recherche de la protéinurie. La signification théorétique de ces différences est discutée. La présence simultanée d'une réaction négative ou légèrement positive des tampons colorimétriques et forte ou modérément positive de la réaction de Heller du test de chaleur de Purdy et de la réaction avec l'acide sulfosalicylique est un signe en faveur de la présence de protéines urinaires du groupe des gamma-protéines.

Zusammenfassung

Es werden vier häufig verwendete Laboratoriumsmethoden zum Nachweis abnormer Globuline im Urin von 19 Patienten mit Plasmocytom oder Makroglobulinämie verglichen. Es ergaben sich Unterschiede zwischen Albumin, Gamma-Globulin und 12 M-Komponenten des Urins in der Beeinflussung eines Indikatorstreifens zum Nachweis der Proteinurie. Deren theoretische Bedeutung wird diskutiert. Die Kombination eines negativen oder nur leicht positiven Tests mit Kolorimeter-Streifen und eines stark oder mäßig positiven Ausfalles der Hellerschen Probe, des Purdyschen Hitzetestes oder der Sulfosalicylsäure-Probe spricht für das Vorliegen von Proteinen aus dem Gamma-Bereich im Urin.

References

1. Ackland, K. and Lyngby, T: Albustix for testing proteinuria. Scand. clin. Lab. Invest. *11* 172 (1959).
2. Bence Jones, H.: On a new substance occurring in the urine of a patient with mollities ossium. Phil. Trans. *138* 55 (1848).
3. Carne, S. Bedside test for proteinuria. Brit. med. J. *1* 399 (1958).
4. Clark, W. M. The Determination of Hydrogen Ions, 3rd ed., p. 179 (Baillière, Tindall and Cox, London 1928).
5. Feigl, F. Spot Tests in Organic Analysis, 5th ed., p. 401 (Elsevier, Amsterdam 1956).
6. Foster, P. W., Rice, J. J. and Wolfson, W. Q. Studies in serum proteins. VI. The extension of the standard biuret method to the estimation of total protein in the urine. J. Lab. clin. Med. *39* 618 (1952).
7. Fraser, S. C. Laboratory trial of paper strip test for proteinuria. Brit. med. J. *i*, 981 (1958).
8. Gornall, A. G.; Bardawill, C. J. and David, M. M.: Determination of serum proteins by means of the biuret reaction. J. biol. Chem. *177* 751 (1949).
9. Grassmann, W. und Hannig, K. Ein quantitatives Verfahren zur Analyse der Serum-Proteine durch Papierelektrophorese. Hoppe-Seylers Z. physiol. chem. *290* 1 (1952)
10. Harrison, G. A. Chemical Methods in Clinical Medicine, 2nd ed., p. 23 (Churchill, London 1937).
11. Heremans, J. F. Les Globulines Sériques du Système Gamma. Leur Nature et leur Pathologie (Masson, Paris/Arscia, Bruxelles 1960).
12. Jencks, W. P.; Jetton, M. R. and Durrum, E. L. Paper electrophoresis as a quantitative method. Biochem. J. *60*: 205 (1955).
13. Korngold, L. and van Leeuwen, G. Multiple myeloma proteins. I. Immunological studies. Cancer *9*: 183 (1956).
14. MacGregor, A. G. A paper strip test for proteinuria. Brit. med. J. *ii*: 430 (1958).
15. Nietzki, R. and Burckhardt, Ed. Über chinoide Derivate des Phenolphtaleins. Ber. dtsch. chem. Ges. *30*: 175 (1897).
16. Putnam, F. W. and Miyake, A. Proteins in multiple myeloma. VII. The free amino groups of Bence-Jones proteins. J. biol. Chem. *227* 1083 (1957).
17. Scheidegger, J. J. Une micro-méthode de l'immuno-électrophorèse. Int. Arch. Allergy *7* 103 (1955).
18. Shaffer, L. and Orr, R. O. Determination of Bence Jones protein in the urine. J. amer. med. Ass. *173* 1137 (1960).
19. Sørensen, S. P. L. Enzymstudier. II. Om Maalingen og Betydningen af Brintionkoncentrationen ved enzymatiske Processer. Medd. Carlsberg Lab. *8*: 1 (1909–10).
20. Stevenson, G. T. Detection in normal urine of protein resembling Bence Jones protein. J. clin. Invest. *39*: 1192 (1960).
21. Todd, J. C. and Sanford, A. A. Clinical Diagnosis by Laboratory Methods, 9th ed., p. 92 (Saunders, Philadelphia 1943).

Author's address: Dr. J. K. Smith, Cornell [illegible] Dept. of Internal Medicine, Bellevue Hospital, *New York*, N.Y. (USA).

Acta haemat. 30: 155–158 (1963)

Aus der I. Medizinischen Klinik und Poliklinik der Universität Mainz (Direktor: Prof. Dr. R. Duesberg)

Über die Bindung löslicher Antigen Antikörper Komplexe an Erythrocyten

Von F. Gramlich und H. E. Müller

Wie Untersuchungen von Sorkin (1) und Weigle (2) zeigten, sind zelluläre Blutbestandteile an der Eliminierung von Antigen-Antikörper Komplexen aus dem Blutkreislauf wesentlich beteiligt. Für Leukozyten und Thrombozyten wurde in diesem Zusammenhang nachgewiesen, daß sie lösliche, im Blutplasma entstandene Antigen-Antikörper Produkte entweder phagozytieren oder an ihrer Oberfläche adsorbieren und dabei Schädigungen erfahren, die zur Zellzerstörung Anlaß geben (3 4).

Die vorliegende Arbeit soll einen Beitrag zu der Frage liefern, welche Rolle die Erythrozyten bei der Eliminierung von löslichen Antigen-Antikörper Komplexen zu spielen vermögen. Dabei lag die von Duesberg (5) geäußerte Vorstellung zugrunde, daß den Erythrozyten auf Grund des multivalenten Bindungsvermögens ihrer großen Gesamtoberfläche und damit ihrer möglichen Adsorptionsfähigkeit *eine Reinigungsfunktion im strömenden* Blut zukommt. Wie in früheren Arbeiten gezeigt werden konnte, ist die Adsorption an die Erythrozytenoberfläche ein durchaus spezifischer Vorgang, was an körperfremden, makromolekularen Substanzen wie etwa Bakterien-Lipopolysacchariden (6) oder Myxoviren (7) ebenso gezeigt werden konnte wie an körpereigenen, homologen Plasmaproteinen (8, 9 10, 11)

Im Folgenden wurde mit einer Technik, die schon bei der Untersuchung des normalen Plasmaproteinfilmes an der Oberfläche menschlicher Erythrozyten zur Anwendung gelangte (9) geprüft, in welchem Ausmaß eine Adsorption von löslichen Antigen-Antikörper Komplexen an die Oberfläche von Kaninchen-Erythro-

zyten erfolgt. Diese löslichen Immunkomplexe wurden stets im Antigen-Überschuß hergestellt. Als Modellsystem diente Human-Albumin und Kaninchen-Antikörper. Dabei war in den Versuchsansätzen einmal das Antigen, zum andern der Antikörper mit ^{131}Jod markiert. Als Vergleich dienten die Adsorptionsquoten der Einzelbestandteile des Immunkomplexes (^{131}Jod-markiertes Human-Albumin und ^{131}Jod-markiertes Kaninchen-gamma-Globulin).

Material

1. ^{131}Jod-markierter Kaninchen-Antikörper gegen Human-Albumin wurde nach folgender Methode hergestellt: Kaninchen wurden mit Freunds Adjuvans gegen Human-Albumin (Behring-Werke, Marburg) sensibilisiert. Blut und antikörperhaltiges Serum wurde durch Herzpunktion gewonnen. In 1,75 m Ammonsulfat-Lösung wurde das Kaninchen-gamma-Globulin gefällt, in Sörensen-Phosphatpuffer p_H 8,0 gelöst und nach einer leicht modifizierten Methode von Friedman und Sternemann (12) mit ^{131}Jod (Radiochemical Centre, Amersham) markiert. Aus der so behandelten Lösung wurde im Äquivalenzpunkt der nephelometrischen Heidelberger-Kurve, der durch ein Trübungsmaximum angezeichnet ist (13, 14), mit der berechneten Albumin-Menge ein spezifisches Albumin-Antialbumin-Antikörper-Präcipitat ausgefällt, wiederholt mit physiologischer NaCl-Lösung ausgewaschen, um freie Jod-Anteile weitgehend zu entfernen, in 0,01 n HCl aufgelöst und auf einer Säule mit Sephadex G 100 (Säulenhöhe 65 cm) in Albumin und Antikörper aufgetrennt (15, 16). In den ersten Fraktionen reichert sich das Antikörper-Protein an, das in Lösung rein vorliegt, nachdem sich das stets in geringen Mengen vorhandene Albumin nach Neutralisieren mit Antikörper unter Präcipitatbildung niedergeschlagen hat. Aus den letzten Fraktionen konnte nach Neutralisieren ein löslicher Antigen-Antikörper-Komplex gewonnen werden, dessen gamma-Globulin-Antikörper-Gehalt aus den gemessenen Impulszahlen der Aktivitätsmessung errechnet wurde. Methodisch bedingt erhält man zusammen mit einem derartigen Antigen-Antikörper-Komplex stets überschüssiges Albumin. Rein formal wurde bei der sicher anzunehmenden 2-Wertigkeit des Antikörper-Moleküls (17) pro Molekül gamma-Globulin zwei Moleküle Albumin angesetzt, so daß der lösliche Antigen-Antikörper-Komplex aus 1 Molekül Antikörper und 2 Molekülen Antigen dargestellt wird.

2. Unmarkiertes Human-Albumin *trocken* wurde von den *Behringwerken, Marburg*, erhalten.

3. ^{131}Jod-markiertes Human-Albumin stammte vom *Radiochemical Centre*, Amersham; es wurde zur Entfernung des freien Jods vor Verwendung 24 Stunden bei 4° C gegen physiologische NaCl-Lösung dialysiert.

4. Die Herstellung von löslichem Antigen-Antikörper-Komplex geschah durch Zusatz der nach einer Heidelberger-Kurve errechneten Menge antikörperhaltigen Kaninchen-Serums zu dem ^{131}Jod-markierten Human-Albumin. Auch hierbei muß mit freiem Albumin, das nicht im Antigen-Antikörper-Komplex gebunden ist, gerechnet werden.

Methode

1. 5 ml so hergestellter Antigen-Antikörper-Komplexlösungen mit bekanntem Gehalt an Immun-Komplexen (Tab. 1, Spalte 4), bei denen einmal der Antikörper und zum andern das Antigen mit ^{131}Jod markiert waren und außerdem von reinen ^{131}Jod-markierten Antikörperlösungen ohne Albumin und von Albuminlösungen ohne Antikörper wurden mit 2 ml einer Erythrocytensuspension vom Kaninchen 2 Stunden

Tabelle I

Die spezifische Oberflächenaktivität eines Immunkomplexes im Vergleich zu seinen Einzelbestandteilen.

1	2	3	4	5	6	7	8
Nr	Protein	Gleichgewichtsmenge	Absolutmenge an Protein	Zahl der Erythrozyten	Protein an der Ery-Oberfläche	Moleküle am Ery	Anreicherungsfaktor
		% P_0	mg Q_a	x10 Z_E	gamma G_{EE}	M_{EE}	F
1	^{131}Jod-	0,0749	30	2,0	22,47	9667	
2	Human-	0,0800	30	2,0	24,00	10320	
3	Albumin	0,0884	30	1,5	26,52	15153	
Mittelwert						11780	
4	^{131}Jod-	0,2230	30	1,5	66,90	38227	3,25
5	Human-Albumin	0,3426	30	1,5	102,78	58728	4,99
6	Kaninchen-	0,2730	30	2,0	81,90	35234	2,99
7	Antikörper	0,5733	30	1,5	173,26	99001	8,40
8	Komplex	0,6314	30	1,5	189,42	108234	9,19
9	^{131}Jod-Kaninchen-gamma-Globulin-Antikörper	*1 746*	*16,3*	*0,9*	*284,85*	*121395*	
10	Human-	4,406	147,8	0,9	1338,24	1472605	12,13
11	Albumin-^{131}Jod-Kaninchen-Antikörper Komplex	2,808	147,8	0,9	2400,99	938.05	7 43

bei Raumtemperatur inkubiert und dann mit einer Sörensen-Phosphatpufferlösung, pH 7,2, die im Verhältnis 1,1 mit physiologischer NaCl-Lösung versetzt war nach folgender Methode ausgewaschen. Die 5 ml Gesamtblutlösung wurden mit 5 ml Pufferlösung vermischt, 3 min bei 1200 g zentrifugiert und vom Überstand jeweils wieder 5 ml abpipettiert. Dieser Waschvorgang wurde 10 mal wiederholt. Die Strahlungsaktivitäten in den einzelnen Waschüberständen und in den Erythrocytensedimenten wurden gemessen. Dazu diente ein Szintillationszähler mit Bohrlochkristall der Firma Frieseke & Höpfner Erlangen.

Ergebnis

Als Gleichgewichtsmenge Po (Tab. I Spalte 3) in % der Ausgangsmenge des betreffenden Proteins wurden die Aktivitäten und damit bei bekannter Relation zwischen Aktivität und Proteingehalt auch die Absolut Menge an Proteinen bezeichnet, die sich dann ergaben, wenn im Überstand und am Erythrozytensedement (das stets auch noch einen Teil Überstand enthielt) *gleiche Impulszahlen*

erreicht waren. Daraus konnte entsprechend dem Schema einer vorhergegangenen Arbeit (9) die Zahl der Moleküle, bzw der Immunkomplexe pro Einzelerythrozyt (M_{EE} Tab. I Spalte 7) aus der Zahl der Erythrozyten im Ansatz (Z_E Tab. I Spalte 5) unter Kenntnis der eingesetzten Gesamtproteinmenge (G_B Tab. I Spalte 4) errechnet werden. Der Anreicherungsfaktor F ergibt sich als Quotient aus der Zahl der Einzelmoleküle Human Albumin oder Kaninchen-gamma Globulin und der entsprechenden Zahl der Immunkomplexe, die an die Erythrozytenoberfläche unter gleichen Bedingungen adsorbiert werden. Wie aus Tab. I hervorgeht, wurden relativ stark differierende M_{EE} Zahlen zwischen den Proben Nr 1–3 und 9 bzw 4–8 und 10–11 gefunden. Das muß in der methodisch bedingten Tatsache gesehen werden, daß bei den Untersuchungen in Nr 1–8 ^{131}Jod markiertes Human Albumin vom Radiochemical Centre, Amersham, Verwendung fand dagegen in den Proben 9–11 Human Albumin der Behringwerke, Marburg. Es zeigten sich bei dem ^{131}Jod markierten Human Albumin aus Amersham beträchtliche Denaturierungserscheinungen, die bei dem entsprechenden Behringwerk Albumin nicht in so starkem Ausmaß gefunden werden konnten. Wie Kontrolluntersuchungen zeigten, ist die Adsorption von Proteinen wesentlich von ihrer Nativität bedingt*. Da aber stets nur die Proben 1–3 mit 4–8 und 9 mit 10–11 verglichen und entsprechende Anreicherungsfaktoren errechnet werden spielt das differierende Verhalten keine Rolle bei den vorliegenden Betrachtungen.

Diskussion

Das Ergebnis läßt erkennen daß sowohl das heterologe Albumin als Antigen wie auch das homologe gamma-Globulin als Antikörper in Form eines Immunkomplexes stärker an die Erythrozytenoberfläche gebunden werden als die entsprechenden frei gelösten Proteine Der gebildete lösliche Antigen Antikörper Komplex besitzt also gegenüber der Erythrozytenoberfläche eine größere Adsorptionsneigung, die als spezifische Oberflächenaktivität definiert ist (18) als die einzelnen Proteine, aus denen sich der Immunkomplex zusammensetzt. Wie aus Tab. I hervorgeht, ist sie um den Faktor 3–12 größer als bei den Bestandteilen, aus denen sich der lösliche Komplex aufbaut. Aus diesem Befund läßt sich mit großer

---

* Unveröffentlichte Versuche

Wahrscheinlichkeit die Schlußfolgerung ziehen, daß die Erythrozyten innerhalb des strömenden Blutes eine Reinigungsfunktion bezüglich gebildeter löslicher Antigen-Antikörper-Komplexe erfüllen. Ihre Fähigkeit zu einer spezifischen Adsorptivbindung solcher Stoffe kann als Beweis für ihre entsprechende Aufgabe betrachtet werden. Ob dabei die Korpuskeln selbst eine Schädigung erleiden und einer beschleunigten Sequestrierung anheimfallen, wie das insbesondere bei den *allergisch hämolytischen Zuständen augenfällig* ist, muß offen bleiben.

Der Deutschen Forschungsgemeinschaft danken wir für ihre Unterstützung. Frl. E. Bettendorf und Frl. R. Sorr danken wir für wertvolle Mitarbeit.

Zusammenfassung

Die Erythrozytenoberfläche adsorbiert lösliche Antigen-Antikörper-Komplexe in stärkerem Ausmaß als die beiden Bestandteile, aus denen sich dieser Immunkomplex zusammensetzt. Als Modellsystem eines löslichen Antigen-Antikörper-Komplexes wurde ein Humanalbumin-Antihumanalbumin-Kaninchenantikörper untersucht. Aus der spezifischen Anreicherung des Immunkomplexes an der Erythrozytenoberfläche wird der Schluß gezogen, daß die Erythrozyten bei der Eliminierung von Antigen-Antikörper-Komplexen aus der Blutbahn aktiv beteiligt sind.

Summary

The surface of erythrocytes adsorbs soluble antigen-antibody complexes to greater extent than it does either of the two components of the complex separately. Human albumin and rabbit antihuman-albumin antibody was investigated as model of soluble antigen-antibody complex. The specific concentration of the immune complex at the red cell surface suggests that these cells are actively involved in the removal of antigen-antibody complexes from the circulation.

Résumé

La surface des érythrocytes adsorbe un complexe soluble antigène-anticorps dans une proportion plus forte que les deux facteurs dont se compose le complexe. Le complexe anti-corps de lapin albumine humaine-antialbumine humaine est étudié comme modèle d'un complexe soluble antigène-anticorps. Sur la base de l'enrichissement spécifique du complexe immunologique à la surface érythrocytaire les auteurs concluent que les érythrocytes jouent un rôle actif dans l'évacuation des complexes antigène-anticorps du sang circulant.

Literatur

1. Sorkin, E.: The Cellular Uptake of Antigens and Their Intracellular Fate. Mechanisms of Antibody Formation, p. 61 (Publ. House Czechosl. Acad. Sci., Prague 1960).
2. Weigle, W. O.: The Elimination of Heterologous Proteins from the Blood of Animals. Mechanisms of Antibody Formation, p. 33 (Publ. House Czechosl. Acad. Sci., Prague 1960).

3. Miescher, P.: Die experimentellen Grundlagen der Immunologie der Leukozyten und Thrombozyten. Die Immunhämatologie der Leukozyten und Thrombozyten in Miescher und Vorlaender: Immunopathologie in Klinik und Forschung (Thieme, Stuttgart 1961).
4. Miescher, P. und Straessle, R.: Experimentelle Studien über den Mechanismus der Thrombozyten-Schädigung durch Antigen-Antikörper Reaktionen. Vox sang. 1: 83 (1956).
5. Dennig, R.: Abwehrfunktion der Erythrozyten. Schweiz. med. Wschr. 43: 1208 (1960).
6. Lüderitz, O.; Westphal, O.; Sievers, K.; Kröger, E.; Neter, E. und Braun, O. H.: Über die Fixation von P^{32}-markiertem Lipopolysaccharid (Endotoxin) aus E. coli an menschliche Erythrozyten. Biochem. Z. 330: 34 (1958).
7. Burnet, F. M. and Stanley, W. M.: The Viruses; in Anderson: Hemagglutination by Animal Viruses (Academic Press, New York/London 1959).
8. Müller, H. E. und Gramlich, F.: Untersuchungen am natürlichen Serumproteinfilm der Erythrozytenoberfläche. Acta haemat. 29: 155 (1963).
9. Müller, H. E. und Gramlich, F.: Die normale Zusammensetzung des Plasmaproteinfilmes an der Oberfläche normaler menschlicher Erythrozyten. Hoppe-Seylers Z. physiol. Chem. (im Druck).
10. Müller, H. E. und Gramlich, F.: Ergänzende Untersuchungen zum Plasmaproteinfilm an der Oberfläche normaler menschlicher Erythrozyten. Naturwissenschaften 50: 447 (1963).
11. Hughes-Jones, N. C. and Gardner, B.: The exchange of ^{131}J-labelled lipid and ^{131}J-labelled protein between red cells and serum. Biochem. J. 83: 404 (1962).
12. Pressman, D. and Sternberger, L. A.: The relative rate of jodination of serum components and the effect of jodination on antibody activity. J. amer. chem. Soc. 72: 2226 (1950).
13. Kabat, E. A. and Mayer, M. M.: Experimental Immunochemistry (C. C. Thomas, Springfield, Ill. 1961).
14. Schultze, H. E. und Schwick, G.: Quantitative immunologische Bestimmung der Plasmaproteine. Behringwerk-Mitt. 35: 1 (1958).
15. Gramlich, F.; Morando, D. und Müller, H. E.: Die Reindarstellung von Antikörpern durch Gelfiltration. Naturwissenschaften 49: 431 (1962).
16. Morando, D. und Müller, H. E.: Physikochemische Untersuchungen an einem rein dargestellten Antikörper. Hoppe-Seylers Z. physiol. Chem. (im Druck).
17. Müller, H. E. und Gramlich, F.: Zur Valenz von präzipitierenden Antigen-Antikörper Systemen. Z. Hygiene 148: 282 (1962).
18. Wolf, K. L.: Physik und Chemie der Grenzflächen (Springer, Heidelberg 1957).

Adresse der Autoren: Dr. F. Gramlich und Dr. H. E. Müller, I. Medizinische Klinik und Poliklinik der Universität, Langenbeckstr. 1, Mainz (Deutschland).

Acta haemat. 30 159-167 (1963)

From Medical Department C, Copenhagen County Hospital, Glostrup
(Chief Dr P From Hansen)

Histochemical Studies of Leukocytes from an Inflammatory Exudate

V Alkaline and Acid Phosphatases and Esterases*

By Henrik R. Wulff

In the present study migrating granulocytes and macrophages obtained by the skin window" technique were examined histochemically for activity of alkaline and acid phosphatases (phosphomonoesterases) and esterases. Activity of these enzymes has previously been investigated in leukocytes from peripheral blood, but migrating cells have received little attention.

Methods

In each experiment successive populations of migrating leukocytes were studied using Rebuck's skin window technique (11 15) The leukocytes migrated from small excoriation to coverslip, which was changed hourly for 12 hours. After removal from the lesion the coverslips were dried for up to 6 hours (or for up 2 days in the alkaline phosphatase series) fixed in suitable fixative, rinsed in tap water incubated in the specific medium, rinsed, counterstained with Mayer's haematoxylin and mounted in glycerine jelly The incubation media (see below) were prepared and filtered immediately before use.

Principle of histochemical reactions: The enzymes are demonstrated using simultaneous coupling azo dye techniques, and the incubation media contained naphthol (or substituted naphthol) ester diazonium salt and buffer maintaining an optimum pH. The enzyme in question hydrolyses the ester and the liberated naphthol groups react in situ with the diazonium salt forming an insoluble coloured precipitate in the cytoplasm of the positive cells.

Alkaline phosphatase reaction (4 skin window experiments) The incubation medium contained sodium α-naphthyl phosphate and Fast Garnet GBC salt in propanediol buffer pH 8.75 (3). The coverslip preparations were fixed in acetone at 0 °C for 30 to 60 minutes before incubation for 60 minutes at 4 °C (6) or for 20 to 30 minutes at room temperature.

Acid phosphatase reaction (4 skin window experiments) The incubation medium contained sodium α-naphthyl phosphate (1 mg/ml) and Fast Garnet GBC salt (1 mg

---

Aided by grant from Arvid Nilsson Fond"



Fig. 1) Alkaline phosphatase reaction in migrating leukocytes from 12 hour preparation. The neutrophils show varying alkaline phosphatase activity whereas the macrophage is negative. b) Acid phosphatase reaction in migrating leukocytes from 11 hour preparation. Neutrophils and macrophages are equally positive and contain numerous granules. Inset: Macrophage and neutrophil from control preparation incubated in the acid phosphatase medium without substrate. The cells are negative apart from few large precipitates of decomposed azo dye.

ml) in acetate buffer (0.1 N, pH 5.0) (8). The coverslip preparations were fixed in acetone at 0 °C for 30 minutes or in 10% neutral formol-saline for 5 to 10 minutes before incubation for 30 to 45 minutes at 37 °C.

α-Naphthyl acetate method for non-specific esterases (4 skin window experiments). The incubation medium contained α-naphthyl acetate and Fast Blue B salt in phosphate buffer pH 7.4 (2). The preparations were fixed in 10% neutral formol-saline at 4 °C for 10 minutes before incubation for 30 minutes at room temperature. A few preparations were incubated in medium containing β-naphthyl acetate.

Naphthol AS-D chloroacetate method for non-specific esterases (6 skin window experiments). The incubation medium contained naphthol AS-D chloroacetate and Fast Garnet GBC salt in barbital buffer pH 7.4 (7). The preparations were fixed for 30 seconds in neutral formalin 10% in methanol at 0 °C (or for 5 minutes in neutral formol saline at 4 °C) before incubation for 30 minutes at room temperature. To ascertain the specificity of the reactions a number of coverslips were incubated in the various media

omitting the specific substrate. The different histochemical reactions were also applied on blood films. The erythrocytes washed off, when the blood films were fixed in cold acetone or neutral formol saline, but the leukocytes remained adherent. The skin window experiments were performed on two healthy subjects.

Results

Each of the phosphatase and esterase reactions presented a characteristic distribution of enzyme activity

Alkaline phosphatases In the skin window preparations the migrating neutrophils presented a varying alkaline phosphatase activity The cell clusters contained both negative and positive neutrophils, the reaction in the positive cells ranging from a few brown granules to a dense granular precipitate (fig 1 a) The average alkaline phosphatase activity was strongest in the neutrophils from late "skin window" preparations. The eosinophils were negative and basophils were not recognised in these nor in the other series The macrophages were also negative or contained few granules (fig 1 a The control preparations were negative. The neutrophils in blood films also showed varying activity but none of the cells were as positive as the most strongly positive migrating cells. All lymphocytes, monocytes and eosinophils were negative.

Acid phosphatases Activity of these enzymes was demonstrated in all types of migrating leukocytes. Neutrophils, eosinophils and macrophages were equally positive and there was no appreciable variation in the staining intensity of the individual cells (fig 1 b) The migrating leukocytes from the control preparations contained irregular yellow crystals of decomposed azo dye, which, however were quite different from the small distinct brown granules in the other preparations (fig 1 b) Neutrophils, eosinophils and monocytes from blood films were moderately positive, and some lymphocytes contained a few granules. In blood films incubated in the control medium, however the non-specific precipitates sometimes had a similar distribution.

Esterases. The two methods for non-specific esterases revealed different activity patterns. When *α-naphthyl acetate* was used as substrate all granulocytes were negative, but the macrophages were positive as shown by a diffuse black precipitate in their cytoplasm (fig 2) In the blood films the monocytes stained intensely (fig 2) whereas lymphocytes and granulocytes were negative The control preparations were negative, and no esterase activity was observed,

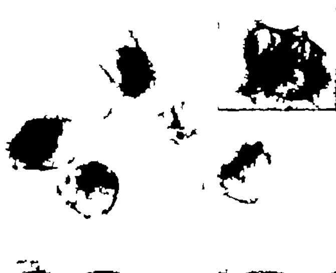


Fig 4. α-Naphthyl acetate reaction for non-specific esterases in migrating leukocytes from 12 hour preparation. The macrophages are positive and the neutrophils negative. No nuclear counterstain. Inset Positive monocyte from peripheral blood, same reaction.

when β-naphthyl acetate was used as substrate The *naphthol AS-D chloroacetate* method revealed a strong esterase activity in neutrophilic granulocytes. At the onset of migration the cytoplasm of the neutrophils presented a very intense red staining whereas the reaction was slightly weaker in the large cell clusters from late preparations (fig 3a and b) Due to the diffuse staining of the neutrophilic cytoplasm the previously described process of cytoplasmic budding and the presence of free cytoplasmic globules were easily seen in these preparations (17) The first macrophages to migrate only presented a very weak staining but in the late preparations some of the macrophages contained coarse granules or large inclusions of intensely stained material (fig 3b and c) The eosinophils presented no esterase activity with this method and no staining was seen in the control preparations. In the blood films the neutrophils were intensely stained the eosinophils and the lymphocytes were negative and the monocytes were weakly diffusely stained or negative

Comment

The previous histochemical studies were concerned with a number of well-defined enzymatic processes and the findings were



Fig. 3. Naphthol AS-D chloroacetate reaction for non-specific esterases.) Intensely positive migrating neutrophils from 3 hour preparation. Three cytoplasmic globules can be seen. No nuclear counterstain. b) Two weakly positive macrophages and three strongly positive neutrophils from late "skin window" preparation. Counterstained with haematoxylin.) Macrophages from 12 hour preparation containing heavily stained inclusions. Counterstained ith haematoxylin.

discussed in terms of leukocyte metabolism (17 18, 19 20) The present results are more difficult to interpret, due to the fact that the metabolic role of phosphatases and esterases in leukocytes is largely unknown. Their substrate specificity is low and possibly each of the histochemical reactions demonstrates several enzymes, which are capable of hydrolyzing the synthetic substrate However it seems of interest that the various types of migrating leukocytes present completely different patterns of phosphatase and esterase ctivity

Earlier reports on the phosphatase and esterase activity of leukocytes are mainly concerned with cells from peripheral blood. The alkaline phosphatase reaction has been accepted as routine method in clinical haematology and need no further comment (3, 21). The α-naphthol esterase reaction in monocytes was first described by BRAUNSTEINER (2) and MOLONEY et al., applied the naphthol AS-D chloroacetate method to blood films and marrow smears. They also reported that tissue histiocytes are strongly positive (7). These observations have been confirmed in few more recent papers (1 4). RABINOVITCH AND ANDREUCCI employed GOMORI lead phosphate technique for the demonstration of acid phosphatases in blood films (10), and later RHEUCK et al. used

the same method for the staining of "skin window" preparations (12). The technique, however, is unsatisfactory as the results are difficult to interpret due to marked diffusion of the reaction product. Recently Loffler and Berghoff have described an azo dye technique for the demonstration of phosphatases at pH 6.3 to 6.5 (5), but it seems doubtful, whether this method is able to demonstrate the same enzymes as the usual acid phosphatase reactions, which are performed at pH 5. The standard azo dye method, which was used in the present study, proved very successful for the demonstration of acid phosphatases in "skin window" preparations, but was less satisfactory when applied to blood films.

The most characteristic feature of the alkaline phosphatase reaction in migrating neutrophils (and in neutrophils from blood films) was the pronounced variation in the activity of the individual cells. In the "skin window" preparations the neutrophils constituted a mixture of negative, weakly and strongly positive cells. Similar variations have previously been observed in the case of phosphorylase and glucose-6-phosphate dehydrogenase, whereas all the other enzymes studied presented almost the same activity in all neutrophils apart from gradual variations in successive "skin window" preparations (17, 18, 19, 20). It is possible that some enzymatic processes vary with the changing activity of the individual neutrophils, whereas others are concerned with their basic metabolism. In this connection it is interesting that the alkaline phosphatase and phosphorylase activity of blood neutrophils varies under pathological conditions (3, 16, 21), whereas for instance esterase activity recorded by the naphthol AS-D chloroacetate method presents no such changes (4). The alkaline phosphatase activity in neutrophils from "skin windows" has previously been studied by Rebuck et al. and Perillie and Finch, who found that the activity in migrating neutrophils varies with that of blood neutrophils under pathological conditions (9, 12, 13).

It is well known that the non-specific esterases represent a heterogenous group of enzymes, but it has been subject to discussion, whether the different histochemical methods demonstrate the same enzymes (2, 7). However, the observation that the α-naphthyl acetate and naphthol AS-D chloroacetate methods reveal completely different patterns of activity shows that these substrates are not hydrolysed by the same enzymes. In addition no leukocyte esterases were able to hydrolyse β-naphthyl acetate.

In all the reactions the precipitation of the azo dye complex was confined to the cytoplasm of the leukocytes, but otherwise it is doubtful, whether the azo dye techniques are able to reveal the

correct intracellular localisation of the enzymes. The distinct granular precipitates, which were observed with the acid phosphatase reaction, may however reflect a lysosomal or mitochondrial localisation of the enzymes (8) Some of the macrophages from late "skin window" preparations contained large inclusions which stained intensely with the naphthol AS-D chloroacetate method. It is likely that these inclusions were ingested fragments of neutrophilic cytoplasm, the presence of which is frequently seen in May-Grünwald-Giemsa stained preparations.

The controversial problem of the origin of the macrophages has had a central position in earlier studies of the migration in "skin windows" The large number of macrophages in late preparations can only be explained by a haematogenous origin, but it is not known whether they are migrated lymphocytes, monocytes or both. Morphologically most of the macrophages resemble monocytes, but REBUCK AND CROWLEY describe a progressive alteration in their size and shape, which they interpret as a gradual transformation of lymphocytes into large macrophages (11) RIIS did not confirm this sequence, but found that those macrophages, which did not *spread over the surface of the coverslip, resembled lymphocytes (14)* The morphological evidence for the lymphocytogenous origin of macrophages in "skin windows" is, however inconclusive. The present experiments show that histochemically the macrophages are more closely related to the monocytes, particularly in respect to α-naphthol esterase activity which was only demonstrated in these cell types. This observation suggests that the macrophages are migrating monocytes.

Summary

Activity of alkaline phosphatases, acid phosphatases and esterases was studied histochemically in migrating leukocytes from "skin windows" using azo dye coupling techniques. Esterases were demonstrated both by the α-naphthyl acetate method and the naphthol AS-D chloroacetate method. The different types of migrating leukocytes presented different patterns of activity Leukocytes from blood films were examined using the same methods, and a noticeable similarity was observed between the enzymatic pattern of exudative macrophages and blood monocytes. The experiments also showed that the two esterase methods do not demonstrate the same enzymes.

Résumé

Étude histochimique de l'activité des phosphatases alcalines et acides des leucocytes émigrés dans une fenêtre cutanée à l'aide d'une technique de copulation avec un colorant azoté. Recherche des esterases par la méthode de l'α-naphthylacétate

ainsi que par celle de naphthol-AS-D-chloracétate. Les divers espèces de leucocytes émigrés montrent des activités différentes. Les mêmes méthodes ont été appliquées à l'étude de leucocytes de frottis du sang périphérique; une analogie frappante entre les activités enzymatiques des macrophages de l'exsudat et de celles des leucocytes du sang pourrait être observée. Les recherches démontrent en outre que les deux méthodes de recherche des esterases ne déterminent pas les mêmes ferments.

Zusammenfassung

An ausgewanderten Leukozyten eines »Hautfensters« wurde mit einer Azofarbstoff-Kupplungsmethode die Aktivität der alkalischen und sauren Phosphatasen und der Esterasen histochemisch untersucht. Die Esterasen wurden sowohl mit dem α-Naphthylacetat als auch mit dem Naphthol-AS-D-Chloracetat Verfahren nachgewiesen. Die verschiedenen Arten ausgewanderter Leukozyten zeigten verschiedene Aktivitäten. Mit denselben Methoden wurden Leukozyten in Blutausstrichen untersucht, und es fand sich eine auffallende Ähnlichkeit der enzymatischen Befunde bei Exsudat Makrophagen und bei Blut Monozyten. Die Versuche ergaben ferner daß mit den beiden Esterase-Methoden nicht dieselben Enzyme nachgewiesen werden.

References

1. Báguena Candela, R. y Forteza Bover, G. El cloroacetato de naftol AS-D para la demostración de la actividad de las esterases inspecificas en los leucocitos. Rev clin. esp. 82. 324 (1961).
2. Braunstein, H. Esterase in leucocytes. J. Histochem. Cytochem. 7. 202 (1959).
3. Hayhoe, F G J and Quaglino, D. Cytochemical demonstration and measurement of leucocyte alkaline phosphatase activity in normal and pathological states by modified azo-dye coupling technique. Brit. J Haemat. 4: 375 (1958)
4. Löffler, H. Cytochemischer Nachweis von unspezifischer Esterase in Ausstrichen. Klin. Wschr 39. 1220 (1961)
5. Löffler, H. und Berghoff W. Eine Methode zum Nachweis von saurer Phosphatase in Ausstrichen. Klin. Wschr 40. 363 (1962)
6. Merker, H. und Hausmeyer, L. Die alkalische Phosphatase neutrophiler Leukozyten. Dtsch. med. Wschr 85. 233 (1960)
7. Moloney W C McPherson, K. and Fliegelman, L. Esterase activity in leukocytes demonstrated by the use of naphthol AS-D chloroacetate substrate. J Histochem. Cytochem. 8. 200 (1960)
8. Pearse, A. G E. Histochemistry Theoretical and Applied (Churchill, London 1960)
9. Perillie, P E. and Finch, S. C. Alkaline phosphatase activity of exudative leukocytes in acute leukaemia. Blood 18. 572 (1961)
10. Rabinovitch, M. and Andreucci, D. A histochemical study of "acid" and alkaline phosphatase distribution in normal human bone marrow smears. Blood 4 580 (1949)
11. Rebuck, J W and Crowley J H. A method of studying leukocytic functions in vivo. Ann. N Y Acad. Sci. 59. 757 (1955)
12. Rebuck, J W Monto R W Monaghan, E. A. and Riddle, J M. Potentialities of the lymphocyte with an additional reference to its dysfunction in Hodgkin disease Ann. N. Y Acad. Sci. 73. 8 (1958).
13. Rebuck, J W Petz, A. J Riddle, J M.; Priest R. J and LoGrippo, G. A: Human leukocytic functions in the tissues, in CIBA Found. Study Group No. 10: Biological activity of the leucocyte (Churchill, London 1961).

14. Riis, P The Cytology of Inflammatory Exudate (Munksgaard, Copenhagen 1959)
15. Riis, P and Wulff H. R.: Dynamic studies of the relation between intravascular and inflammatory neutrophils in normal subjects. Acta haemat. 23: 276 (1960).
16. Takeuchi, T; Tadokoro, N and Ide, H.: Histochemical study on glycogen synthesis from glucose 1-phosphate in blood cells under normal and pathological conditions. J Histochem. Cytochem. *10*: 572 (1962).
17 Wulff H. R. Histochemical studies of leukocytes from an inflammatory exudate. Glycogen and phosphorylase. Acta haemat. *28*: 86 (1962).
18. Wulff H. R. Histochemical studies of leukocytes from an inflammatory exudate. II. Succinic dehydrogenase, mitochondrial α-glycerophosphate dehydrogenase and di- and triphosphopyridine nucleotide diaphorases. Acta haemat. *29*: 208 (1963)
19. Wulff H. R.: Histochemical studies of leukocytes from an inflammatory exudate. III. Di- and triphosphopyridine nucleotide-linked dehydrogenases. Acta haemat. *30*: 16 (1963)
20. Wulff H. R.: Histochemical studies of leukocytes from an inflammatory exudate. IV Uridine diphosphate glucose—glycogen transglycosylase. Acta haemat. *30*: 123 (1963).
21. Wulff H. R. and Riis, P Cytokemisk påvisning af basiske fosfataser i leukocytter Ugeskr Laeg. *123*: 1503 (1961).

Author's address: Dr. H. R. Wulff, Medical Dept. C, Copenhagen County Hospital, *Glostrup* (Denmark).

Acta haemat. 30 168–180 (1963).

Department of Pathology Southmead Hospital, Bristol

Bridge Anticoagulant Neutralizing Agent: Properties and Isolation

By F Nour Eldin

Frank and Hartman (4) and Govaerts and Gratia (5) demonstrated that the blood-clotting time of haemophilic blood was improved by the addition of a small volume of normal plasma. The responsible agent, associated with the plasma globulin (11 24) was termed antihaemophilic globulin (9) The preparation of potent material combined with the development of new laboratory techniques, have recently lead to the re assessment of the beneficial effect of this antihaemophilic factor (factor VIII) Of the many interesting observations which have accumulated, two are noteworthy the rapid turn-over of antihaemophilic globulin (1 8) and the need for extraordinary amounts of this factor in order to achieve appreciable correction of the deficient plasma thromboplastin (12, 23) Nour Eldin (16 18) showed that the first phenomenon was not peculiar to haemophilia, being also observed in normal individuals. On the other hand, evidence has been provided showing that the presence of a specific blood-clotting inhibitor Bridge anticoagulant reduces the *in vivo effect* of factor VIII (22, 23) Following the latter work it was speculated that should Bridge anticoagulant be removed or its activity be neutralized it would be possible to attain a marked improvement in the formation of plasma thromboplastin in haemophilic blood by using relatively small amounts of factor VIII

Recent investigations (17 19) have demonstrated that an agent which seems to stem from thrombin-fibrinogen interaction, renders inert Bridge anticoagulant, hence the absence of the latter in serum.

Further experiments were carried out in an endeavour to isolate this neutralizing agent from fibrinogen thrombin mixtures sub-

jected to different conditions (unpublished work). Whilst it was apparent that fibrino-peptide (10) was devoid of such an effect, contamination with thrombin made it difficult to assess the significance of the slight activity of other preparations.

The present work provides evidence showing that Bridge anti-coagulant neutralizing agent (BANA) is present in sera obtained by the coagulation of normal blood and stored pooled plasma. The preparation and separation of BANA from these sources are described and its properties reported.

Methods

Reagents were prepared as described by Nour-Eldin (19, 21). Although the present work was carried out using platelet suspension, practically the same results are obtained on utilizing insoluble phospholipid as platelet-substitute (giving substrate clotting-time of 9–10 seconds with normal reagents). For maximal stability solutions of human factor V and porcine factor VIII were made in cold (4° C) buffered saline, consisting of equal volumes of 0.9% NaCl and imidazole buffer pH 7.3. These were freshly prepared and kept in an ice-bath until added to the incubation mixture.

Elution from barium sulphate was carried out by a solution consisting of 2 volumes of 2.5% sodium citrate and 8 volumes of 0.9% NaCl.

Ethanol fractionation: The method of Cohn et al. (2) was essentially followed.

Blood-clotting tests: Since the blood-clotting times and prothrombin consumption are within normal range in 58% and 30% respectively of patients with haemophilia and Christmas disease, no particular importance was attached to the results of these tests. On the other hand, plasma thromboplastin formation being always deficient, offered the best method to detect improvements in these defects. The procedure used was as follows.

Plasma thromboplastin activity: The basic incubation mixture in most instances consisted of equal volumes of undiluted haemophilic $Al(OH)_3$-treated plasma, imidazole buffer pH 7.3, factor VIII solution, normal serum and 0.05 M $CaCl_2$. The activity of plasma thromboplastin formed after 6 minutes at 37° C was tested by taking 0.1 ml thereof and adding it together with 0.1 ml 0.025 M $CaCl_2$ to 0.1 ml substrate (normal citrated plasma) at 37° C, recording the clotting time. Certain modifications in the procedure and concentration of reagents were, however, necessitated by the purpose in view. Details of these are given in the appropriate sections.

Results

Effect of undiluted serum. Table I shows that the use of undiluted normal serum (in place of the usual 1 : 10 dilution) in the incubation mixture (containing undiluted haemophilic plasma) improves the clotting-time of the substrate, whether or not factor VIII was incorporated in the mixture. Serum, however, had no effect when utilized in incubation mixtures free of Bridge anticoagulant (containing no haemophilic plasma) as indicated by the results in table

Table I

The effect of undiluted serum on the generation of plasma thromboplastin from undiluted haemophilic plasma.

Reagent in incubation mixture	MI			
	1	2	3	4
Serum	1 10	undiluted	1 10	undiluted
Al(OH)$_3$-haemophilic plasma (undiluted)	+	+	+	+
Factor VIII (0.3 mg/ml)	+	+	—	—
Imidazole buffer pH 7.3	+	+	+	+
0.9% NaCl	—	—	+	+
Platelet suspension	+	+	+	+
0.03 M CaCl$_2$	+	+	+	+
Substrate clotting-time	28	15½	72	56½

Six minutes after adding CaCl$_2$ 0.1 ml of the mixture and 0.1 ml 0.025 M CaCl$_2$ were simultaneously added to 0.1 ml normal citrated plasma (substrate) at 37 C. Results are verage of 4 experiments in each case.

+ = used — = not used.

Table II

Absence of serum action on mixture free of Bridge anticoagulant.

Exp.	Factors in incubation mixture (equal volumes)*				Substrate clotting time, sec.**
	Factor V (20 mg/ml)	Factor VIII (0.3 mg/ml)	Platelet suspension	Serum	
A	+	—	+	1:10	56
B	+	—	+	undiluted	57
C	+	+	+	1 10	10
D	+	+	+	undiluted	10

Mixed with imidazole buffer pH 7.3 and 0.025 M CaCl$_2$ in all experiments.
For details see table I.

II. This denotes that serum specifically counteracts the inhibitory action of Bridge anticoagulant.

Three conceivable mechanisms by which serum could exert this action were considered a) accelerating thrombin formation from prothrombin, b) the effect of Bridge anticoagulant is counter acted by an increase in the concentration of factor IX and/or factor VII and c) inactivating Bridge anticoagulant by a hitherto unknown agent. The first probability was excluded by the experiments illustrated in table III from which it will be seen that serum fails to shorten the clotting time of the substrate when added to the latter together with already formed plasma thromboplastin. The following investigations dismiss excess serum factor IX or factor VII from being the responsible agents thus leaving the third mechanism as being the most likely explanation.

Table III

Absence of serum action on substrate.

Procedure	Reagents	Experiment 1	2	3	4
1. *Incubation mixture*	Normal serum (undiluted)	+	—	+	—
Equal volumes of	Normal serum (1:10)	—	+	—	+
reagents incubated	Undiluted $Al(OH)_3$-treated				
at 37° C for 6 min.	haemophilic plasma	+	+	+	+
	Factor VIII (0.3 mg/ml)	+	+	—	—
	Platelet suspension	+	+	+	+
	Imidazole buffer pH 7.3	+	+	+	+
	0.05 M $CaCl_2$	+	+	+	+
2. *Testing activity*					
0.1 ml of incubation					
mixture together with	1:6 serum in 0.025 M $CaCl_2$*	—	+	—	+
0.1 ml of reagent	0.025 M $CaCl_2$	+	—	+	—
(opposite) added to					
0.1 ml substrate					
3. *Result***					
Clotting time of					
substrate, sec.		14	27	36	68

* Prepared by mixing 1 volume of undiluted serum with 5 volumes of 0.03 M $CaCl_2$.
** The substrate clotting-time (sec.) in each case is the average of 4 tests.
+ = used; — = not used

Table IV

The effect of different sera on plasma thromboplastin production from undiluted haemophilic plasma.

Undiluted serum	Specimen A	B	C
	substrate clotting time, sec.**		
Normal	14	13	–
Haemophilic	27	28	28
Christmas disease	28	26½	26½
Control (1:10 normal serum)	27½	27	27½

Serum was incubated at 37° C with equal volumes of undiluted $Al(OH)_3$-treated haemophilic plasma, Factor VIII (0.3 mg/ml), factor V solution (10 mg/ml imidazole buffer pH 7.3), platelet suspension and 0.05 M $CaCl_2$. The activity of formed plasma thromboplastin was tested after 6 minutes.
Normal citrated plasma was used as substrate. Results are averages of 2 experiments in each case.

Activity of different sera. Sera prepared from the blood of patients with haemophilia or Christmas disease, who have prothrombin consumption within normal range (in order to avoid high thrombin concentration in the incubation mixture) were utilized. The results demonstrated in table IV reveal that these sera, in contrast to the normal reagent, are ineffective in improving the deficient

plasma thromboplastin formed in the presence of undiluted $Al(OH)_3$-treated haemophilic plasma, thus denoting that neither excess factor VII or X (in both haemophilic and Christmas disease sera) nor high levels of factor IX (in haemophilic serum) is the responsible agent.

The effect of dilution. Table V shows that although different samples of undiluted serum (obtained from different normal subjects) show great variations in their action on the inhibitory effect of haemophilic plasma, slight degrees of dilution have marked adverse effect on this property in all instances.

The effect of ether. During attempts to identify the ether-precipitated fraction (7) containing the responsible agent, a curious observation was that whilst the majority of BANA activity was associated with the globulin fraction the total activity after ether treatment exceeded that of the original serum. It was, therefore, decided to investigate the effect of freezing in the presence of excess ether (13). Promising results were obtained on mixing with 50% ether and lowering the temperature: an increase in the activity amounting to 4–8 fold was achieved at temperatures below —60° C (acetone-CO_2 mixture). This can be seen on comparing the results before and after ether treatment at —25° C and —65° C (table VI). Since simple freezing at these temperatures did not influence the activity of serum, it was concluded that the presence of ether during freezing was essential for the enhancement of BANA activity. No further increase in the activity of serum was noticed on repeating the ether treatment.

Properties of BANA. Plasma separated from expired bank blood and freeze-dried pooled plasma were found, after being converted to serum, to be suitable sources of BANA. The latter could

Table V

The effect of dilution on serum activity

Normal serum	A	B	C	D	E
	Substrate clotting time, sec.				
Undiluted	11	12	11	14	15
1:2	14	15	14½	17	18
1:4	17½	19½	18½	22½	22
1:10	19½	23	25	28	27

Serum diluted as indicated was incubated with the same reagents as in table IV. The same source of haemophilic plasma was used in all experiments. Normal citrated plasma was used as substrate. Results are average of 2 experiments in each case.

Table VI

Effect of ether-treatment on serum activity

Normal serum*	Before	Ether treatment	
		After	
		25 °C	–45 °C
	Substrate clotting time, sec.		
Undiluted	11½	9	9
1:2	13	11	9
1:4	16	12	10
1:8	19½	14	12½
1:16	25½	18	15½
Control (0.9% NaCl)	27	–	–

Serum treated and diluted as indicated was added to an incubation mixture consisting of equal volumes of $Al(OH)_3$-treated undiluted haemophilic plasma, platelet suspension, normal serum (1:10), Imidazole buffer pH 7.3 and Factor VIII (0.3 mg/ml). Six minutes after adding 0.05 M $CaCl_2$ at 37 °C the activity of formed plasma thromboplastin was tested, using normal citrated plasma as substrate.

be adsorbed on calcium phosphate, aluminium hydroxide and barium sulphate. For quantitative elution, however, barium sulphate proved to be superior (80 % recovery) to the other reagents (30 to 40 %). Satisfactory elution from barium sulphate can be attained with 0.5 % trisodium citrate in 0.8 % NaCl. At this concentration of sodium citrate negligible amounts of factors VII and IX are eluted. Precipitation of BANA from solution occurred at 50 % saturation with ammonium sulphate and 1.1 M phosphate. No deterioration in serum BANA was observed on storing sera at –25 °C for 4 months, at 2 °C for 1 week, at 37 °C for 16 hours or at 50 °C for 10 minutes. On the other hand, serum became practically devoid of activity following incubation at 60 °C for 5 minutes. Freeze-drying had no appreciable effect on the activity of serum, but caused a slight loss in that of isolated BANA.

Preliminary clinical trial of serum. Although this paper is mainly concerned with the identification and separation of BANA, a concise note on the preliminary in vivo evaluation of this substance in serum does not appear to be out of place. It will, however, be appreciated that these preliminary trials were limited by the restricted availability, at the time, of sterile serum which also being untreated with ether contained low activity of BANA.

For prophylactic effect, observations were conducted on 5 patients aged 4–18 years who have seldom been free of spontaneous bruises and suffered frequent attacks of haemarthrosis. On being given approximately 200 ml. serum at intervals of 9–17 days, the spontaneous bruises completely disappeared and all expressed that

haemarthrosis became less frequent, though not completely prevented. They were also less liable to develop intramuscular haematomas following minor trauma. After approximately 2 years, without the patients being aware, the serum was changed to plasma in 3 cases. The clinical change was remarkable all 3 patients developed spontaneous bruises and the severe attacks of haemarthrosis recurred within 4–10 weeks. In the remaining 2 patients, serum was combined with plasma these remained free of subcutaneous bleeding and had negligible swelling of joints. After a further period of 14 months, serum was discontinued and these 2 patients received double volumes (400 ml) of plasma. This was followed within 4–8 weeks by noticeable haemorrhagic episodes.

Therapeutically serum was tried on 3 occasions.

1 A male aged 13 years with Christmas disease, bled excessively after circumcision at the age of 8 months. He has also suffered repeated attacks of haemarthrosis in the elbows and knees. Haematuria occurred once after a blow on the right loin. Severe bleeding in the left knee followed a fall. On admission to another hospital, his left lower limb was put in plaster which had to be removed 2 days later because of intolerable pain. He was then provided with calliper and advised not to stand on this leg. Two years later I saw him with greatly swollen left knee which could not be extended or flexed. Rest to the joint with back splint for 10 weeks, reduced the size of the joint which, however remained stiff and bent at an angle of 140 Given serum and plasma transfusions, the deformity was corrected under general anaesthesia and plaster pplied for 9 weeks. The resulting severe muscle wasting hindered the patient from using his leg without caliper. An intensive physiotherapy course was, therefore, instituted together with serum and plasma transfusions at 9–14 days intervals no complications being encountered following the extensive passive and active manipulations carried out. After 11 weeks the patient was able to walk and attend Grammar School without the aid of the calliper stick or any other ppliance.

2. A boy with Christmas disease, aged 8 years, was given 700 ml serum before and during the extraction of 4 teeth, but excessive bleeding continued. The administration of 200 ml plasma was followed by instant cessation of bleeding. No ppreciable loss of blood was recorded after these transfusions.

3. A man aged 76 years with Christmas disease, was admitted for the extraction of 4 teeth. Family history showed that he was one of 4 brothers who all suffered from spontaneous haemarthrosis and severe bleeding after teeth extractions. His elbows were fixed at 150 and he had swollen knees and ankles. Teeth removal was preceded by serum and plasma transfusions the operative field was remarkably free of bleeding and post-operative loss of blood was negligible.

Changes in the blood-clotting defect. Concurrently with the above clinical observations, laboratory tests were performed in order to study the effect on the coagulation mechanism of serum and/or plasma administered in cases of haemophilia and Christmas disease. Blood samples examined were procured before and 10 minutes after the transfusions using in the latter case another vein in order to avoid contamination with the injected material. The results in two of these cases are presented in table VII No concrete conclusions are derived from the alterations in the blood-clotting time and prothrombin consumption index (see methods) these being given for completion of record only It is, however obvious that maximal improvement in the production of plasma thrombo-

plastin in the presence of undiluted $Al(OH)_3$-treated plasma is induced by combined serum and plasma transfusion. However for adequate haemostasis during operative procedure, substrate clotting-time well below 20 seconds is necessary. This would require larger amounts of these reagents.

Table VII

Effect of in vivo serum and plasma on the blood-clotting tests in haemophilia and Christmas disease.

Patient	Transfusion (equal volumes)	Test: Blood clotting time, min.	Test: Prothrombin consumption index %	Test: Thromboplastin formation, substrate clotting time in sec.
Haemophilia	Nil	>30	200	105
	plasma + saline	23	75	70
	plasma + serum	19	50	50
Christmas disease	Nil	>30	180	92
	serum + saline	18½	30	59
	plasma + saline	17	50	68
	plasma + serum	14½	25	39
	plasma + plasma	13½	25	51

Total volume approximately 400 ml. Plasma and serum separated from normal donor' blood. The effect of reagents was tested on specimens of blood procured 10 minutes after completion of transfusion, usually lasting 1–2 hours.

Using patient's undiluted $Al(OH)_3$-treated plasma in the incubation mixture with equal volumes of patient' serum 1 10, platelet suspension, imidazole buffer pH 7.3 and 0.03 M $CaCl_2$. The activity of formed plasma thromboplastin was tested after 6 minutes at 37 C on normal substrate (citrated plasma).

From these investigations, it was concluded that serum or serum and plasma, but not plasma alone reduce the incidence of *spontaneous* bleeding, particularly subcutaneous haematomas. For the management of trauma and surgery however larger volumes of serum must be supplemented by the appropriate blood-clotting factor (VIII or IX) in sufficient amounts. This being in conformity with in vitro experiments prompted the isolation and concentration of BANA for use in major operations and systematic, large-scale clinical trial. The results of these will be described in a separate article. However the method used for isolating BANA is now described.

Isolation of BANA

The method consists of three main steps preparation of serum, activation with ether and precipitation of activated material.

I. Preparation of serum In the case of fresh blood, serum is obtained from blood allowed to clot at 37 C for 3 hours and stored thereafter at 4 C for 16 hours. For pooled plasma, of the several methods tried (in order to obtain the maximal amount of serum and remove the voluminous clot) the following method, being applicable for aseptic work, is now adopted. T each 100 ml plasma, 7 ml M $CaCl_2$ is added. The specimen

which usually clots in 25–40 minutes is incubated at 37° C for 4–5 hours and at 4° C for 16–18 hours. The vessel containing the clotted plasma is then stored at —25° C for 24 hours. On thawing, the clot which is now reduced in size permits the decantation of 80–90 ml serum from each 100 ml original plasma.

2. Activation with ether. After cooling serum to 1° C, 0.5 volume of cold (1° C) ether is added. Mixing is carried out by rapid rotation of the container for 3–4 minutes at 0° C. The mixture is then freezed in, and allowed to reach the temperature of an acetone-CO_2 bath. The frozen material is allowed to thaw slowly at 4–6° C. Thawing at temperatures above 20° C is detrimental to the activity of BANA. After centrifugation at 5° C, the serum layer is separated and freed of ether under vacuo.

3. Isolation of activated BANA. Although adsorption on barium sulphate and elution can be used, ethanol fractionation (2) is more suitable for large-scale aseptic work. BANA activity is concentrated in the protein fraction precipitated at pH 5.8, 0.66 M fraction ethanol and 0.04 M sodium concentration. γ-globulin is extracted from this by a reagent containing 0.05 M fraction ethanol, 0.005 M Na and 0.6 M glycine at pH 5.5. The remaining precipitate containing BANA is dissolved in 0.9% NaCl. After removing undissolved material by centrifugation at 4° C freeze-drying is carried out.

Activity and stability of freeze-dried BANA. Material purified by elution from barium sulphate with 0.5% sodium citrate had no significant effect on the blood-clotting time of normal blood and did not materially alter the one-stage prothrombin time of factor V or factor VII deficient plasma. On the other hand, it corrected the clotting-time and prothrombin consumption of haemophilic and Christmas disease blood. Tested by the thromboplastin generation method, it improved the action of factors VIII and IX in the presence of undiluted haemophilic or Christmas disease plasma respectively. This is illustrated in table VIII which also shows the effect of storage (under vacuo, over P_2O_5) on the activity of freeze-dried BANA. Slight loss of activity occurred after 2 months. Longer periods of storage resulted in an appreciable loss, amounting in some cases to about 60% after 6 months.

Table VIII

Stability of freeze-dried BANA.

Concentration of BANA mg/ml buffered saline*	Specimen No. (10/6) fresh	(10/6) stored*	(15/4) fresh	(15/4) stored
	Substrate clotting time, sec.			
3.0	11½	13	11½	14
1.5	15	17½	15½	20
0.75	21	23	22	25½
Control (buffered saline)	28	27½	28½	27

Buffered saline consists of equal volumes of 0.9% NaCl and imidazole buffer pH 7.3.

Specimen 10/6 was stored for 2 months and specimen 15/4 for 4 months under vacuo over P_2O_5.

One volume of BANA solution was mixed at 37° C with equal volumes of undiluted $Al(OH)_3$-treated haemophilic plasma, factor VIII solution (0.3 mg/ml) imidazole buffer pH 7.3 and platelet suspension. Six minutes after adding 0.05 M $CaCl_2$, the activity of plasma thromboplastin was tested by adding 0.1 ml thereof together with 0.1 ml 0.025 M $CaCl_2$ to 0.1 ml substrate (normal citrate plasma) at 37° C, recording the clotting-time.

Discussion

There is no dispute regarding the absence of factor VIII activity in serum. Nevertheless, undiluted normal serum is effective in improving the plasma thromboplastin formed in the presence of undiluted haemophilic plasma and augments the correction produced by factor VIII (table I) Examination of certain explanations for this action of serum demonstrated the specificity against the inhibitory effect of Bridge anticoagulant. The conclusion that none of the known blood-clotting factors is responsible for Bridge anticoagulant neutralization receives further support from differences in properties. For the successful recovery of factors VII and IX from barium sulphate, a higher concentration of sodium citrate is required. Factors XI and XII are both unaffected by adsorbents and the latter is stable at 60 C for 30 minutes (25 26)

The exact method by which ether enhances the activity of BANA in serum is at present difficult to define. None of the layers discarded after ether treatment had an inhibitory effect. The removal of an anticoagulant by ether is, therefore, most unlikely Direct activation of already-formed BANA appears to be a remote possibility since the activity of material isolated from untreated serum was not increased after ether-treatment. Treatment with chloroform induces proteolytic activity in plasma (3) while certain agents release biologically active peptides from plasma and serum (27) Some of these e. g acetone, dilution, versene, epsilon-aminocaproic acid and foreign surfaces, have recently (6, 14 15 28) been shown to be dependent on the presence of one of the blood-clotting factors, Hageman factor (factor XII) These observations suggest the following possibilities, which are being investigated for potentiating BANA activity in serum by ether a) ether may release from serum proteins a compound with a similar activity to BANA or which facilitates its action, and b) BANA exists in serum as a part of a complex compound from which a more active agent is split off by ether

Studies on defibrination (19) have denoted that BANA can be furnished by low concentrations of exogenous thrombin. In the light of this information, defective thrombin generation cannot be accepted as a reason for the absence of BANA in haemophilic and Christmas disease sera. It is, therefore, suggested that this absence is most probably due to the utilization of BANA in the inactivation

of the inhibitor in these conditions. This view is supported by the presence of BANA in the sera of two cases of combined factor VIII deficiency and capillary defect (VON WILLEBRAND's syndrome) the blood being free of Bridge anticoagulant (29, 30).

The destructive effect of undiluted serum on plasma thromboplastin (20) being counteracted by excess factor V in undiluted haemophilic plasma as well as added factor V is of no importance in the interpretation of the present experiments.

In order to overcome the inhibitory effect of plasma with a high level of Bridge anticoagulant, at least 2 volumes of normal serum are required. The clinical use of comparable volumes would be beset with difficulties. However the use of pooled plasma from expired blood as a source of serum together with the activation of BANA with ether at low temperature, considerably eases the situation.

Acknowledgements. I wish to thank all the patients, blood donors and Hospital Staff whose co-operation made possible the testing of BANA; the United Manchester Hospitals for a grant from their Research Endowment during the initial stages of the work and, Mrs. CHRISTINE ELDER and Mrs. ANGELA FOX for typing the manuscript.

Summary

Normal undiluted serum contains Bridge anticoagulant neutralizing agent (BANA), the action of which is independent of the already-known blood-clotting factors in serum. The properties of, and the effect of certain conditions on this agent have been studied. A method is described for its separation, from fresh blood and stored plasma, after being activated with ether at low temperature. In vitro experiments as well as preliminary clinical trial showed that for physiological correction and adequate improvement of the haemophilic and Christmas disease defects, the addition of BANA to factors VIII and IX respectively is necessary.

Résumé

Le sérum normal, non dilué, contient un facteur qui peut neutraliser l'anticoagulant Bridge (BANA). Son action est indépendante de tous les facteurs de la coagulation du sérum connus jusqu'à présent. Les propriétés et le mode d'action de ce facteur sont étudiés. Une méthode d'isolation à partir du sang frais et du plasma conservé après activation par l'éther à des basses températures est décrite. Des expériences in vitro et une étude clinique provisoire démontrent que l'addition du BANA aux facteurs VIII et IX est nécessaire pour une correction physiologique et une amélioration adéquate des défauts de coagulation de l'hémophilie et de la christmas disease.

Zusammenfassung

Normales unverdünntes Serum enthält ein Agens, das das Bridge-Anticoagulans neutralisiert (BANA). Seine Wirkung ist unabhängig von den bisher bekannten Gerin-

nungsfaktoren des Serums. Eigenschaften und Wirkungsmodus dieses Agens wurden untersucht. Es wird eine Methode zu seiner Isolierung aus Frischblut und konserviertem Plasma nach Aktivierung mit Äther bei niedrigen Temperaturen beschrieben. Versuche in vitro und eine vorläufige klinische Prüfung zeigten, daß die Zugabe von BANA zu den Faktoren VIII und IX notwendig ist für eine physiologische Korrektur und adäquate Behebung der Gerinnungsdefekte bei Hämophilie und Christmas disease.

References

1. Brinkhous, K. M.; Penick, G. D.; Langdell, R. D.; Wagner, R. H. and Graham, J. B. Physiological basis of transfusion therapy in hemophilia. Arch. Path. *61* 6 (1956)
2. Cohn, E. J.; Gurd, F. R. N.; Surgenor, D. M.; Barnes, B. A.; Brown, R. K. Derouaux, G.; Gillespie, J. M.; Kahnt, F. W.; Lever, W. F.; Liu, C. H.; Mittelman, R. F Mouton, K.; Schmid, K. and Uroma, E. A system for the separation of the components of human blood Quantitative procedure for the separation of the protein components of human plasma. J. amer. chem. Soc. *72* 465 (1950).
3. Delezenne, C. et Pozerski, E. Action protéolytique de sérum sanguin préalablement traité par le chloroforme. C. R. Soc. Biol. *55*: 690 (1903)
4. Frank, E. und Hartmann, E. Über das Wesen und die therapeutische Korrektur der hämophilen Gerinnungsstörung. Klin. Wschr. *6*. 435 (1927).
5. Govaerts, P. and Gratia, A. Contribution à l'étude de l'hémophilie. Rev. belge Sci. méd. *3*. 689 (1931)
6. Keele, C. A. In Schachter's Polypeptides which Affect Smooth Muscle and Blood Vessels (Pergamon, New York 1960).
7. Kekwick, R. A. and Mackay M. E. The separation of protein fractions from human plasma with ether Spec. Rep. Ser. Med. Res. Counc. Lond., No. 286 (1954)
8. Langdell, R. D.; Wagner, R. H. and Brinkhous, K. M. Antihemophilic factor (AHF) levels following transfusions of blood, plasma and plasma fractions. Proc. Soc. exp. Biol., N. Y. *88*: 212 (1955)
9. Lewis, J. H.; Tagnon, H. J. Davidson, C. S. Minot G. R. and Taylor, F. H. L. The relation of certain fractions of plasma globulins to the coagulation defect in hemophilia. Blood *1* 166 (1946)
10. Lorand L. Fibrino-peptide. Biochem. J. *52*: 200 (1952)
11. Lozner, E. L. and Taylor, F. H. L. The coagulation defect in haemophilia studies of the clot promoting activity associated with plasma euglobulin in hemophilia. J. clin. Invest. *18*: 821 (1939)
12. Macfarlane, R. G. Mallam, P. C. Witts, L. J.; Bidwell, E. Biggs, R. Fraenkel, G. J.; Honey G. E. and Taylor, K. B. Surgery in haemophilia. The use of animal antihaemophilic globulin and human plasma in thirteen cases. Lancet *ii*. 251 (1957)
13. McFarlane, A. S. The behaviour of lipoids in human serum. Nature, Lond. *149*: 439 (1942)
14. Margolis, J. The interrelationship of coagulation of plasma and release of peptides. Ann. N. Y. Acad. Sci. *104* 133 (1963).
15. Margolis, J. and Bishop E. A. Interrelations between different mechanisms of release of biologically active peptides from blood plasma. Nature, Lond. *194* 749 (1962)
16. Nour-Eldin, F. Fixed levels of antihaemophilic globulin in health and disease. Fourth Int. Congr. Biochem., Vienna *10*. 17 (1958).
17. Nour-Eldin, F. The neutralisation of Bridge anticoagulant. Nature, Lond. *184* 1419 (1959).

18. Nour-Eldin, F: Some reflections on the use of antihaemophilic globulin. Proc. VIIth Europ. Congr. Haemat., London, Part II, p. 599 (1959).
19. Nour-Eldin, F. Plasma adsorption and Bridge anticoagulant. Acta haemat. 24: 261 (1960).
20. Nour-Eldin, F: The action of serum on plasma thromboplastin. Acta haemat. 28: 265 (1962).
21. Nour-Eldin, F: The in vivo and in vitro behaviour of clotting factors in blood and tissues. Ann. N. Y. Acad. Sci. (in press).
22. Nour-Eldin, F. and Wilkinson, J. F. Bridge anticoagulant. Brit. J. Haemat. 4: 58 (1958).
23. Nour-Eldin, F. and Wilkinson, J. F. In vivo study on the relation between Bridge anticoagulant and antihaemophilic globulin. Brit. J. Haemat. 4: 292 (1958).
24. Pohle, F. J. and Taylor, F. H. L. The coagulation in hemophilia. The effect of intramuscular administration of a globulin substance derived from normal human plasma. J. clin. Invest. 16: 741 (1937).
25. Ratnoff, O. D. and Colopy, J. E. A familial hemorrhagic trait associated with deficiency of a clot promoting factor of plasma. J. clin. Invest. 34: 602 (1955).
26. Rosenthal, R. L., Dreskin, H. and Rosenthal, N. Plasma thromboplastin antecedent (P.T.A.) deficiency. Blood 10: 120 (1955).
27. Schachter, M. Delayed slow contracting effect of serum and plasma due to release of substances resembling Kallidin and bradykinin. Brit. J. Pharmacol. 11: 111 (1956).
28. Webster, M. E. and Ratnoff, O. D. Role of Hageman factor in the activation of vasodilator activity in human plasma. Nature, Lond. 192: 180 (1961).
29. Wilkinson, J. F., Nour-Eldin, F. and Israels, M. C. G.: Bridge anticoagulant in von Willebrand's syndrome. Lancet ii: 115 (1958).
30. Nour-Eldin, F. Different haemostatic defects in one family. Brit. med. J. 2: 502 (1960).

Author's address: Dr. F. Nour-Eldin, Dept. of Pathology, Southmead Hospital, Bristol (England).

Acta haemat. 30. 181–189 (1963)

Department of Medicine, Laboratory of Coagulation and Proteolysis, University of Louvain

On the Concept of Slowly and Rapidly Acting Coumarin Drugs

By M. Verstraete, J. Hellemans*, C. Vermylen and M. Vorlat

It is well established that after administration of coumarin or indanedione derivatives the activity of prothrombin, factor VII factor IX and factor X decreases in the peripheral blood. This is believed to result from the partial inhibition of the production of the mentioned clotting factors formed in the reticulo-endothelial system. After administration of sufficiently high doses of coumarin drugs to block completely the synthesis of these clotting factors, their subsequent fall in activity will depend on their consumption in vivo, provided that the drug does not interfere directly with the turnover rate.

This study was designed to compare the rapidity of action of three coumarin derivatives in dogs.

Methods

Methods as previously described were used (8).

Five blood samples were taken on the first day of each experiment, and 3 blood samples a day on the following days. The results obtained in the different assays have been converted to percentages of normal plasma activity of the respective clotting factor.

For each experiment and each factor the regression line and correlation coefficient were calculated, the distribution being assumed as normal (1). The values below 15% in the P and P test and below 10% in the factor VII plus X assay were not taken into account. On the curves constructed by plotting logarithm of activity in percentage versus time it could be seen that these plots were situated just above the best fitting straight line. The t-value was calculated from the students test.

The half-life (t ½) is defined as the time interval required for a fall in concentration of a clotting factor activity by 50%. This value is calculated on the regression lines.

---

* Aspirant van het N. F. W. O.

Experimental Procedure

Sixteen dogs (14–30 kg.) were used for 22 experiments with 3-(1'phenylpropyl)-4-hydroxy coumarin (Phenprocoumon) bis-3-(4-hydroxy-coumarinyl)-ethyl acetate (Ethyl-biscoumacetate) or 3-(α-acetonyl-p-nitrobenzyl)-4-hydroxycoumarin (Nicoumalone) A very high loading as well as maintenance dose was given during at least 84 hours in order to obtain rapidly high concentration of drug at the synthesis sites. The exact mode of administration, dosage and choice of coumarin drugs in each experiment is summarised in table I.

Table I

Dose in mg. of coumarin, orally or intravenously (I.) administered in morning (M), afternoon (A) or at night (N)

Number of experiments	Anticoagulant	Mode of administration	Dose first day			Dose following days		
			M	A	N	M	A	N
6	Phenprocoumon	I.	30		10	10		10
1	Phenprocoumon	I.	10		10	–		10
2	Phenprocoumon	oral	30	6	9	9	3	9
3	Phenprocoumon	oral	12	9	9	9	–	9
5	Ethyl-Biscoumacetate	oral	2400	900	900	900	900	900
5	Nicoumalone	oral	32	16	16	16	16	16

The coumarin drugs were kindly supplied by Roche and Geigy (S. A. Christiaens, Brussels) under the brandname Marcoumar Tromexan and Sintrom respectively

Results

In figs. 1–3 the mean calculated regression lines for different clotting factors are represented. The curve corresponding to Phenprocoumon administration (fig 1) is the calculated mean curve for 12 experiments performed in different dogs. For each experiment at least 12 determinations of the different assays were performed at fixed time intervals after the administration of a blocking dose of Phenprocoumon. Each line represents therefore 140 determinations. Nicoumalone (fig. 2) and Ethyl-biscoumacetate (fig 3) have been administered to 5 days respectively The mean half life of the different clotting factors can be seen in table II

Discussion

In addition to the determination of the biological half-life of single blood clotting factors we have also calculated the "half life" of mixtures of blood clotting components according to the values of combined assessments. It is realised that each clotting factor has its own survival time. The disappearance rate of clotting factors

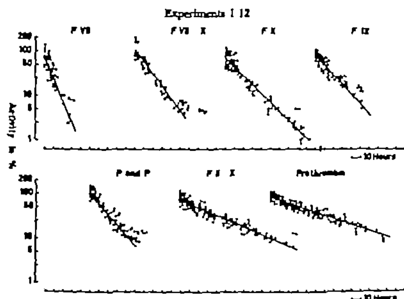


Fig 1 The mean calculated regression lines for different clotting factors in 12 experiments (1–12) with completely synthesis blocking doses of Phenprocoumon during at least 84 hours.

Table II

Half-life of various clotting factors (hours) after administration of massive doses of Ethylbiscoumacetate, Nicoumalone and Phenprocoumon.

	Ethyl-biscoumacetate	Nicoumalone	Phenprocoumon
P and P test	12.8	15.1	13.0
F VII	5.9	6.6	6.2
F VII + X	10.2+	13.5+	11.0
F X	14.5	18.3	16.8
Prothrombin	39.2	38.8	42.9
F IX	12.6	16.7	15.4
F II + X	30.0	38.7	33.4

The differences between these half-life values, found after administration of 3 different coumarin drugs are statistically not significant ($p > 0{,}05$) excepted + (= 2,70).

calculated on the basis of such non-specific determinations is nevertheless of interest (7 10 16) and provides valuable information on the mutual contribution of various clotting factors in the combined assays.

e) Rapidity of action. It is widely accepted that with therapeutic doses, coumarin drugs differ in their rapidity of action on the depression of clotting factors (2, 9 11) The latent period for the

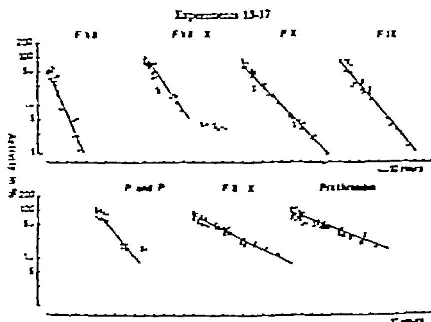


Fig. [illegible] The mean calculated regression lines for different clotting factors in 5 experiments 13-17 with comparatively synthesis blocking doses of [illegible] during about 84 hours.

Table III

Decrease in activity of various clotting factors 4.6 and 10[illegible] hours after administration of [illegible] dose of Ethyl-biscoumacetate 5 experiments and Phenprocoumon (13 experiments) mean values

	Quick-time sec	P and P	F VII	F VII–X	F X	Prothr.	F IX	F II X
Before therapy	6,8	100,9	72,7	113,9	72,3	92,3	1 1	80[illegible]
After 4,6 h range 3–6,5	17	83,3	[illegible]3	85,9	61[illegible]	86,9	[illegible]	73
After 12[illegible] h range 9–11 h	[illegible]3	[illegible]7	23,9	[illegible]4,2	[illegible]0,7	71,3	[illegible]4,5	[illegible]

"rapidly" acting Ethyl-biscoumacetate is between 8–12 hours and for the "slowly" acting Phenprocoumon between 24–48 hours. The time required to produce therapeutic "hypoprothrombinaemia" is said to be 18–[illegible] hours and 48–[illegible] hours respectively (4, 5). During the latent period no change in the concentration of the different clotting factors is expected to occur.

Therefore the linear regression line corresponding to the exponential decrease of the various clotting factors should be above the values determined at 5 and 10 hours. We found however in the

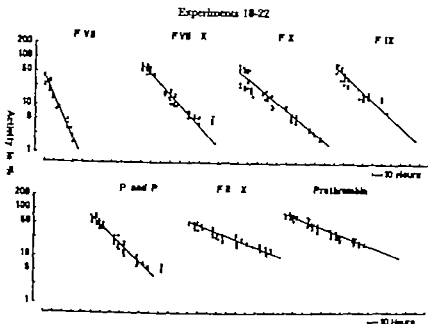


Fig. 3. The mean calculated regression lines for different clotting factors in 5 experiments (18-22) with completely synthesis blocking doses of Ethyl-biscoumacetate during at least 84 hours.

present experiments (fig 1–3) that 5 and 10 hours after massive administration of Ethyl biscoumacetate, Nicoumalone or Phenprocoumon the determined values in the factor VII and combined factors VII plus X assays are on or very close to the linear regression line. The interpretation of these findings is that the coumarin effect is already complete within this short time interval.

In table III the mean values are given of the different assays obtained before, 4 6 and 10.2 hours after the loading dose of Phenprocoumon (15 experiments) or Ethyl-biscoumacetate (5 experiments) After 10 hours, the factor VII and combined factors VII plus X activity is already decreased to respectively 20 and 28 % of the original value, although the one stage prothrombin" time is not significantly changed. It is to be noted that between the 4.6th and 10.2th hours after the administration of the coumarin drug, the factor VII activity decreases by almost 50 %. This time interval corresponds to the half life of factor VII which suggests that ± 5 hours after the intake of coumarin drugs the block on factor VII production is complete although the one-stage "prothrombine"

time still remains within normal limits. It is likely that the synthesis of other coagulation factors is similarly blocked at the same time. Our experiments lead to the observation that after administration of massive doses of the anticoagulant a similar activity pattern is found for Phenprocoumon (administered either by intravenous or oral route) as for Nicoumalone or Ethyl-biscoumacetate (oral administration) The same period of latency which is less than 4.6 hours was found for the drugs tested. Similar results were obtained by PERLICK (13) SMITH (15) and RODMAN (14) but several other authors found a different delay of effect for each anticoagulant tested. This controversy can partly be explained by the different doses used (15) and the various methods selected to follow the coumarin effect. The importance of the sensitivity of the methods is apparent. When P and P values are compared to the one stage prothrombin" times at 10 hours after intake of the loading dose, the former test corresponds to 58.7 / and the one stage prothrombin time is still in the normal range. Furthermore if the factor VII assay is selected, the activity of coumarin drugs on blood coagulation is more rapidly detected than with a one stage prothrombin time on undiluted blood (performed with a factor VII sensitive tissue-thromboplastin)

b) Slope of the curves In figs. 1–3 can be seen that the slope of the different curves remains constant even at low concentrations of the determined clotting factors except for the combined factor VII plus X assays and the P and P test where a systematic deviation of the linearity is observed. This phenomenon can be explained by the observation that a factor VII free plasma still has a 7.5–10 / activity in the combined factors VII plus X assessment (8) This unexpected finding is to be related to the discordant proportions of clotting factors in coumarin plasma whereas in the serial dilutions of the normal plasma, used to establish the reference curves, all clotting factors are similarly diluted. In the beginning of coumarin administration, factor VII disappears very rapidly from the peripheral circulation and this influences the factor VII plus X assay Once the factor VII activity becomes very low a further decrease does not influence significantly the factors VII + X assay provided the factor X activity is still at a moderate level. A similar explanation can be offered for the interpretation of the P and P test which at the start reflects the activity of factors II VII and X, subsequently factors II + X and eventually mainly factor II For factor IX

assessments, less accurate low values are explained by incomplete deficiency in factor IX activity of the substrate plasma used.

c) The difference between slowly and rapidly acting coumarin derivatives The alleged shortest (Ethyl biscoumacetate) and longest acting (Phenprocoumon) coumarin derivatives have been choosen deliberately for these experiments Nicoumalone is considered to take an intermediate position. We have demonstrated in this study that these three anticoagulants have the same period of latency The half life of the various clotting factors influenced by the three anticoagulants does not differ significantly as can be seen in table II. These results were obtained after administration of doses of the anticoagulant capable of completely blocking synthesis of the appropriate factors, which we consider to be the unique method of comparing the rates of induction of effect produced by different coumarin drugs.

In determining the time interval necessary to obtain a one stage "prothrombin time within the therapeutic zone, it has been found that this level was rapidly obtained with Ethyl-biscoumacetate and after some delay with Phenprocoumon (4 5) The effect is more rapidly attained with Nicoumalone than with Dicoumarol (11) or Warfarin (12)

The main difference is that Phenprocoumon is slowly eliminated and a single dose of this drug can have a sustained effect for one week. To decrease the activity of a clotting factor to a certain level, only a small proportion of the cells producing the relevant factor have to be blocked and so the appearance of the coumarin effect will be delayed. With the rapidly excreted Ethyl biscoumacetate a larger dose is needed to obtain an adequate effect by virtue of maintaining the tissue concentration against urinary excretion. Because of this larger dose, more productive cells are blocked and the rate of effect is chiefly dependent on the half-life of the clotting factors in the circulation.

These studies have shown that classification into "rapidly acting" and "slowly acting" anticoagulants drugs can be misleading They are only rapidly acting" or 'slowly acting because by clinical experience induction dosages are used which have been found to bring the patient to the choosen therapeutic level and not beyond. When used in large enough doses they produce this induction effect with equal speed. The kinetics of the decrease in activity of a clotting factor sensitive to coumarin drugs depends on the half life

Varia

Asian and Pacific Society of Hematology

The third congress of the Asian and Pacific Society of Hematology will be held in Jerusalem, Israel, August 17 to 23, 1964.

Requests for further information should be addressed to the secretary of the congress, Prof. G. Izak, P.O.B. 499 Jerusalem, Israel.

Libri

Methoden der immunhämatologischen Forschung. Hgb. von *Carl Steffen*, Bibliotheca Haematologica, Fasc. 14. S. Karger AG, Basel/New York 1963. VI + 202 S., 40 Abb., 42 Tab., Preis DM/sF 52.–

Im Rahmen des achten Kongresses der europäischen Gesellschaft für Hämatologie, der im September 1961 in Wien stattfand, wurde eine Reihe von Kolloquien über immunohämatologische Techniken abgehalten. C. Steffen gebührt das Verdienst, die Ergebnisse dieser Aussprache im vorliegenden Band einem breiteren Kreise zugänglich gemacht zu haben. Die besprochenen Themen umfassen den Coombstest, die Leukoagglutination, den Antiglobulinkonsumptionstest, die passive Hämagglutination, die Immunpräzipitation im Agargel sowie die Sedimentationsanalyse in der Ultrazentrifuge. Alle jenen, die an diesen Methoden interessiert sind, bietet dieser Bericht reichhaltige Anregungen.

A. Hässig, Bern

Ergebnisse der Bluttransfusionsforschung, Bd. VII. Schock und Kollaps – Fehler, Irrtümer und Gefahren bei der Bluttransfusion und Infusion von Blutersatzmitteln – Kolloquien. Bibliotheca Haematologica, Fasc. 16. Hgb. von *H. [illegible]* und *W. Spielmann*. S. Karger AG, Basel/New York 1963. 424 S., 100 Abb., Preis Fr. 60.–

Die Deutsche Gesellschaft für Bluttransfusion hielt vom 23. bis 26. Mai 1962 ihre 10. Tagung unter dem Vorsitz von Prof. Dr. H. Bürkle de la Camp ab. Die Hauptthemen waren erstens Schock und Kollaps und zweitens Fehler, Irrtümer und Gefahren bei der Bluttransfusion und Infusion von Blutersatzmitteln. Nach einführenden Übersichtsreferaten über Pathophysiologie und Klinik verschiedener Schockformen wurden in weiteren Vorträgen unter anderem haematologische Veränderungen bei Traumen, Hexosemonophosphatzyklus im Erythrocytenstoffwechsel, Membranschädigungen menschlicher Erythrocyten und Haemolyse und Plasmaeiweißkörper unter besonderen Bedingungen der Chirurgie besprochen. Unter dem zweiten Thema kamen Fehler und

Gefahren bei Bluttransfusionen und Infusionen von Blutersatzmitteln in serologischer und allergischer Hinsicht, in der Organisation des Bluttransfusionswesen und hinsichtlich der Indikationsstellung von Bluttransfusionen und Infusionen von Blutersatzmitteln zur Diskussion. Außerdem widmeten sich einige Autoren verschiedenen Fragen der Blutkonservierung. Am Schluß der Tagung fanden Kolloquien statt, die sich vor allem mit immunhaematologischen Untersuchungsmethoden, Arzneimittelgesetz und Bluttransfusionswesen und rechtlichen Fragen im Bluttransfusionswesen beschäftigten.

H. Tröler, Basel

G. Schwarz: **Das C-reaktive Protein.** (Fortschritte der Immunitätsforschung, Bd. 5). Dr. Dietrich Steinkopff Verlag, Darmstadt 1963. XII + 68 S., 1 Abb., Preis DM 18.–

Wie der Autor in seinem Vorwort selbst bemerkt, hat er mit dieser Broschüre den Versuch unternommen, «das zusammenzustellen, was bis heute über das C-reaktive Protein bekannt ist». Dieser Versuch ist ihm bestens geglückt. Unter Zitation von 254 Arbeiten gibt er eine knapp gefaßte, aber umfassende Übersicht über die Biochemie, die pathogenetische und klinische Bedeutung dieses «Entzündungsproteins».

A. Hässig, Bern

Symposium on Kaposi's Sarcoma. Kampala (Uganda) 1961. Hgb. von *Lauren V. Ackerman and James F. Murray.* S. Karger AG Basel/New York 1963. 174 S., 119 Abb., 29 Tab., Preis sFr./DM 52.

Das sogenannte «Kaposi-Sarkom», das 1872 durch Moritz Kaposi als besondere Krankheit in Wien erkannt wurde, ist in Europa immer noch eine recht seltene Erscheinung. In Afrika hingegen stellt es beim Eingeborenen eine relativ häufige Affektion dar, ebenso häufig wie z. B. das Kankroid oder das Melanom der Haut; es ist bei Schwarzen zehnmal häufiger als bei Weißen. Diese Tatsache allein rechtfertigte den Gedanken, ein Symposium über diese Krankheit in Zentralafrika zu organisieren, wofür die Internationale Union gegen den Krebs (UICC) die Patenschaft übernommen hat. In der soeben herausgekommenen Monographie werden von Pathologen und Klinikern, an Hand eines außerordentlich großen, meist aus Afrika stammenden Untersuchungsgutes, die zahlreichen noch offenen Fragen erörtert. Man findet insbesondere sehr wertvolle Beiträge über Statistik, klinische Erscheinungen, pathologische Anatomie, Behandlung usw., über Spezialfragen, wie die immer noch nicht restlos abgeklärte Beziehung der Kaposi-Wucherungen zu den Blutgefäßen und über Einzelheiten der Histopathologie. Die letzten Kapitel sind besonders eindrucksvoll, da bis heute eine derartige Fülle von Befunden (Biopsien, Krankengeschichten und Autopsien) wohl kaum zusammengestellt worden ist. Trotz allen sehr detaillierten histologischen und histochemischen Untersuchungen bleibt die Histogenese der großen Spindelzellen, welche für diese Krankheit eminent charakteristisch sind, im Dunkeln; die meisten Autoren sind der Ansicht gewesen, daß sie außerhalb der Blutgefäße entstehen, also nicht endothelialer Herkunft sind (vielleicht Perizyten oder Schwann'sche Zellen). Man findet auch an einzelnen Stellen der Beiträge die Ansicht verschiedener Pathologen über die eigentliche Natur dieser Wucherung; die meisten sind der Meinung, daß die Bezeichnung «Sarkom» nicht korrekt ist. Für alle Kliniker und Pathologen, die sich mit den Problemen der Hautpathologie beschäftigen, stellt diese Publikation eine wahre Fundgrube von Dokumenten dar.

Fred C. Roulet, Basel

Varia

Asian and Pacific Society of Hematology

The third congress of the Asian and Pacific Society of Hematology will be held in Jerusalem, Israel, August 17 to 23, 1964

Requests for further information should be addressed to the secretary of the congress, Prof. G. IZAK, P.O.B. 499 Jerusalem, Israel.

Libri

Methoden der immunohämatologischen Forschung. Hgb. von *Carl Steffen*, Bibliotheca Haematologica, Fasc. 14. S. Karger AG, Basel/New York 1963. VI + 202 S., 40 Abb., 42 Tab., Preis DM/sF 52.–.

Im Rahmen des achten Kongresses der europäischen Gesellschaft für Hämatologie, der im September 1961 in Wien stattfand, wurde eine Reihe von Kolloquien über immunohämatologische Techniken abgehalten. C. STEFFEN gebührt das Verdienst, die Ergebnisse dieser Aussprache im vorliegenden Band einem breiteren Kreise zugänglich gemacht zu haben. Die besprochenen Themen umfassen den Coombstest, die Leukoagglutination, den Antiglobulinkonsumptionstest, die passive Hämagglutination, die Immunpräzipitation im Agargel sowie die Sedimentationsanalyse in der Ultrazentrifuge. Allen jenen, die an diesen Methoden interessiert sind, bietet dieser Bericht reichhaltige Anregungen.

A. HÄSSIG, Bern

Ergebnisse der Bluttransfusionsforschung, Bd. VII Schock und Kollaps – Fehler Irrtümer und Gefahren bei der Bluttransfusion und Infusion von Blutersatzmitteln Kolloquien. Bibliotheca Haematologica, Fasc. 16. Hgb. von *H. Willenegger* und *W. Spielmann*. S. Karger AG Basel/New York 1963. 424 S., 100 Abb., Preis Fr 60.–

Die Deutsche Gesellschaft für Bluttransfusion hielt vom 23. bis 26. Mai 1962 ihre 10. Tagung unter dem Vorsitz von Prof. Dr H. BÜRKLE de la Camp ab. Die Hauptthemen waren erstens Schock und Kollaps und zweitens Fehler Irrtümer und Gefahren bei der Bluttransfusion und Infusion von Blutersatzmitteln. Nach einführenden Übersichtsreferaten über Pathophysiologie und Klinik verschiedener Schockformen wurden in weiteren Vorträgen unter anderem haematologische Veränderungen bei Traumen, Hexosemonophosphatzyklus im Erythrozytenstoffwechsel, Membranschädigungen menschlicher Erythrozyten und Haemolyse und Plasmaeiweißkörper unter besonderen Bedingungen der Chirurgie besprochen. Unter dem zweiten Thema kamen Fehler und

Acta haemat. 30: 193–203 (1963).

Aus der II. Medizinischen Universitätsklinik in Wien
(Vorstand Prof. Dr. K. Fellinger)

Bestimmung der Glucose-6 Phosphatdehydrogenase-Aktivität in den Erythrozyten zur Erkennung von hypo und aplastischer Erythropoese

Von S. Sailer, F. Sandhofer und H. Braunsteiner

Bei Vorliegen einer Anämie sind zwei Fragen zu beantworten 1. Ist die Lebensdauer der Erythrozyten verkürzt? 2. Produziert das Knochenmark genügend Erythrozyten, bzw. ist es in der Lage, auf die bestehende Anämie mit einer Steigerung der Erythropoese zu antworten? Während die erste Frage durch den Nachweis einer Blutung oder durch Hämolysezeichen meist bereits klinisch beantwortet werden kann, ist das Problem der quantitativen Erfassung einer verminderten effektiven Erythropoese (2) für die klinische Praxis bisher nicht befriedigend gelöst.

Der reife Erythrozyt besitzt einen lebhaften Stoffwechsel. Einige der daran beteiligten Enzyme nehmen im Verlauf der physiologischen Alterung des Erythrozyten an Aktivität ab. Dies gilt besonders für die Acetylcholinesterase (15, 16, 17, 13, 22), die Hexokinase (14), die Phosphohexoisomerase (11), die Glyceraldehydphosphatdehydrogenase (6, 7, 8), die Glucose-6-Phosphatdehydrogenase (9, 10, 11, 6, 7, 8, 1, 4, 12), die 6-Phosphogluconatdehydrogenase (9, 10, 11, 1) und die Glutaminoxalessigsäuretransaminase (19, 20, 21, 3, 5). Diese und auch andere Fermente wurden von uns vergleichsweise untersucht (18). Für eine klinische Anwendung erschien uns die Glucose-6-Phosphatdehydrogenase (G-6-PDH) am geeignetsten, weil die Bestimmung methodisch leicht durchführbar und der Unterschied der Enzymaktivität zwischen jungen und alten Zellen sehr groß ist, so daß aus der gemessenen Enzymaktivität gut auf das mittlere Lebensalter der vorliegenden Erythrozytenpopulation geschlossen werden kann.

Das mittlere Lebensalter einer Erythrozytenpopulation ergibt unter Berücksichtigung des Gesamterythrozytenvolumens Hinweise

auf das Ausmaß der effektiven Erythropoese, d. h. der Anzahl Erythrozyten, die pro Zeiteinheit vom erythropoetischen System ausgeschwemmt werden (2)

Zweck der vorliegenden Untersuchung ist es, die Bestimmung der G-6-PDH zur Beurteilung der effektiven Erythropoese bei verschiedenen Blutkrankheiten heranzuziehen.

Methodik

5 ml Heparinblut werden im Verhältnis 2:1 mit 3%igem Dextran (Fa. Knoll, Ludwigshafen) in physiologischer NaCl-Lösung, die mit TRIS-Puffer auf pH 7,4 gebracht wurde, versetzt, einige Male gekippt und eine Stunde bei Zimmertemperatur stehen gelassen. Während dieser Zeit sedimentierten die Erythrozyten in allen Fällen, die Leukozyten und Thrombozyten befanden sich in der überstehenden Phase. Nach Abheberung der überstehenden Flüssigkeit betrug die Leukozytenkontamination der Erythrozytenschicht höchstens 100 mm³. Eine sorgfältige Abtrennung der Leukozyten ist unbedingt notwendig, da die G-6-PDH-Aktivität in den Leukozyten um 2 bis 3 Zehnerpotenzen über der der Erythrozyten liegt. Eine zu große Leukozytenbeimengung erhöht daher die gemessene Aktivität beträchtlich.

Die Erythrozyten werden durch Schütteln gründlich gemischt, 0,1 ml in ein Zentrifugenröhrchen pipettiert, zweimal mit 2 ml gepufferter HANK'scher Lösung (pH 7,4) durch Zentrifugieren 3 Minuten bei 1000 U/Min. gewaschen, 1,0 ml gesättigte Digitoninlösung zugesetzt, 15 Minuten bei 4° C hämolysiert, dann bei 5000 U/Min. durch 10 Minuten das Stroma abzentrifugiert und das Hämolysat sofort zum Test eingesetzt.

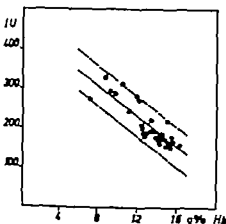
Bestimmung der Fermentaktivität: Der Ansatz mit 0,1 ml Hämolysat, 2,75 ml Triaethanolamin-Puffer ($5 \cdot 10^{-2}$ m + EDTA $5 \cdot 10^{-3}$ m) und 0,1 ml TPN ($1{,}0 \cdot 10^{-2}$ m) wird durch 10 Minuten im durchströmten temperierbaren Küvettenhalter im Photometer *Eppendorf* (Fa. Netheler & Hinz, Hamburg) auf 25° C gebracht, dann mit 0,05 ml Glucose-6-Phosphate-Na ($4{,}1 \cdot 10^{-2}$ m) die Reaktion gestartet. Endvolumen 3 ml, Schichtlänge 1 cm, Wellenlänge 366 nm. Die Reaktion wurde mit Hilfe eines Registriergerätes und eines automatischen Schreibers (Philips PR 2210 A-00) laufend registriert. Die verwendeten Reagentien stammen von der Fa. Boehringer, Mannheim. Die gemessenen Aktivitäten werden in Internationalen Einheiten (I.U. = µMol Substratumsatz/Minute), bezogen auf 10^{10} Erythrozyten, angegeben. Die Bestimmung des Hämoglobins erfolgte nach der Cyan-Hämoglobinmethode.

$$\text{Berechnung: I.U./}10^{10}\ \text{Ery} = \frac{9150 \cdot Hb_V \cdot \Delta E^{366\,nm}_{1\,min}}{Hb_H \cdot Ery}$$

Hb_V = Hämoglobin im Vollblut in g%; Hb_H = Hämoglobin im Hämolysat in g%;
Ery = Anzahl der Erythrozyten im Vollblut mm³ in Mill.

Ergebnisse

1. Normale Enzymaktivität. Bei 44 gesunden Personen mit einem Hämoglobingehalt von 14 bis 16 g% (im Mittel 14,9 g%) im Vollblut wurde die mittlere Enzymaktivität der Erythrozyten untersucht. Sie betrug im Mittel 169,7 I. U. $\sigma = \pm 18{,}4$



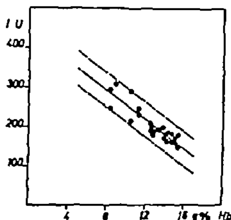
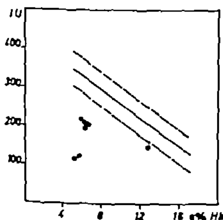


Abb. 1. Funktionell intaktes Knochenmark a) Hämolytische Anämie (und Normale) b) Blutungsanämie ohne Eisenmangel (und Normale).



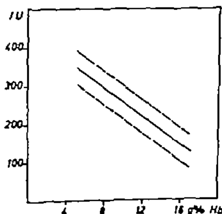


Abb. 2. Hypoplasie ( ) und Aplasie (●) des Knochenmarkes.
Abb. 3. Unbehandelte Perniciosa.

2. *Anämie mit funktionell intakter Erythropoese* In Abb. 1 sind die Enzymaktivitäten der Patienten mit Blutungsanämie ohne Eisenmangel, d. h. nach ausreichender Zufuhr von Eisen, wiedergegeben. Die Aktivitäten sind dabei gegen g / Hb im Vollblut aufgetragen. Es zeigt sich, daß die gemessenen Enzymaktivitäten umso höher liegen, je niedriger der Hb-Spiegel im Blut ist. Es besteht eine sehr gute negative Korrelation zwischen gemessener Enzymaktivität und Hämoglobin im Vollblut ($r = 0{,}937$ $p < 0{,}001$ $y =$

10 Verlaufskurven. Als Maß für die Funktionstüchtigkeit des erythropoetischen Systems wählten wir das Verhältnis der gemessenen Enzymaktivität zum Sollwert bei intakter Erythropoese. In den Abbildungen 9–13 sind einige typische Verlaufskurven der Erythropoese bei Behandlung verschiedener Blutkrankheiten wiedergegeben. Bei einem Fall von Eisenmangelanämie stieg die Ak-

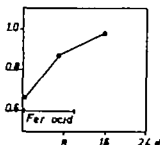


Abb. 9. Eisenmangelanämie.

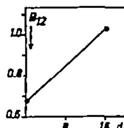


Abb. 10. Perniciosa.

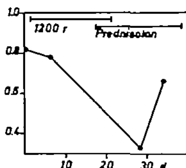


Abb. 11 Lymphadenose.

tivität nach parenteraler *Eisenzufuhr zur Norm an (Abb. 9)* Bei einer unbehandelten Perniciosa normalisierte sich die Aktivität nach 1000 Gamma B_{12} (Abb 10) In Abb. 11 sind die Verminderung der Erythropoese bei einem Fall von chronischer lymphatischer Leukämie unter Röntgenbestrahlung (Gesamtdosis 1200 r) und die darauffolgende Erholung unter Prednisolon dargestellt. In ähnlicher Weise wurde die Erythropoese bei einem Fall von Morbus Hodgkin durch Cytostatica (30 mg TEM) gehemmt. Auch hier konnte durch Prednisolon die Erythropoese gebessert werden (Abb. 12) Im Laufe einer akuten Anurie nahm die Fermentaktivität

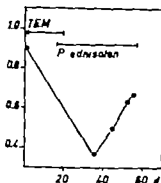


Abb. 12. Morbus Hodgkin.

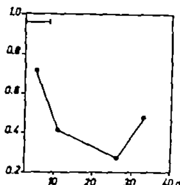


Abb. 13. Anurie.

schon nach wenigen Tagen ab und erreichte ihren tiefsten Wert in der vierten Woche, um dann wieder anzusteigen. Wiederholte Sternalpunktion bestätigten auch morphologisch die mangelnde Erythropoese. Erst in der dritten Woche wurde die Anämie manifest (Abb 13)

Diskussion

In der vorliegenden Arbeit wurde aus der Abnahme der G-6-PDH Aktivität im Laufe der physiologischen Alterung der Erythrozyten auf das mittlere Lebensalter der Erythrozyten einer gegebenen Population geschlossen. Eine normale Fermentaktivität, wie sie bei gesunden Versuchspersonen gemessen wird, entspricht einem normalen mittleren Lebensalter der Erythrozytenpopulation. In diesem Fall halten sich die Zahl der pro Zeiteinheit ausgeschwemmten jungen und die der zugrunde gehenden Erythrozyten die Waage. Die effektive Erythropoese reicht zur Erhaltung des normalen Hämoglobinspiegels aus. Bei Vorliegen einer Anämie bestehen wesentlich kompliziertere Verhältnisse. In diesem Fall bedeutet dieselbe Fermentaktivität, eine normale Lebensdauer der Erythrozyten vorausgesetzt, daß gerade nur so viele junge Zellen ausgeschwemmt als alte Zellen zerstört werden. Dies hat zur Folge, daß die Anämie stationär bleibt und daß, je niedriger der Hämoglobinspiegel ist, pro Zeiteinheit entsprechend weniger Zellen ausgeschwemmt werden. Die Erythropoese ist vermindert und nicht in der Lage, die Anämie zu kompensieren. Liegen die Werte noch tiefer dann reicht die Erythropoese auch zur Aufrechterhaltung des Hämoglobinspiegels nicht aus, so daß die Anämie progredient wird (12) Es ist demnach selbstverständlich, daß zur Beurteilung der effektiven

Erythropoese der aktuelle Hämoglobinspiegel herangezogen werden muß. Aus diesem Grund wird ja auch zur Beurteilung der Erythropoese auf Grund der Retikulozytenwerte empfohlen, diese nicht in Prozentzahlen, sondern in absoluten oder auf die Erythrozytenzahl korrigierten Werte anzugeben (23) Ein Reticulozytenwert von 1% bei 5 Millionen Erythrozyten/mm^3 entspricht beispielsweise einem Wert von 2% bei 2,5 Millionen Erythrozyten/mm^3 In ähnlicher Weise bedeutet bei einer Anämie ein erhöhter Fermentgehalt in den Erythrozyten noch nicht eine gesteigerte Erythropoese.

Ein funktionell intaktes Knochenmark antwortet bei Vorliegen einer Anämie mit einer Steigerung der Erythropoese. Erst eine vom Grad der Anämie abhängige Steigerung der effektiven Erythropoese ist als funktionell intakt zu bezeichnen. Aus diesen Gründen wählten wir die empirisch gefundene Erhöhung der Enzymaktivität bei Anämien mit funktionell intakter Erythropoese als geeignetes Bezugssystem, nach dem die Erythropoese bei verschiedenen Anämien beurteilt werden kann. Auf Grund unserer derzeitigen Kenntnisse ist die Erythropoese bei Blutungsanämien ohne Eisenmangel als funktionell intakt zu bezeichnen, die in solchen Fällen bei verschiedenen Hämoglobinspiegeln gemessenen Enzymaktivitäten wurden daher zur Aufstellung eines derartigen Bezugssystems verwendet. Dabei besteht eine sehr gute negative Korrelation zwischen Fermentaktivität und Hämoglobingehalt des Vollblutes. Bei Werten, die innerhalb der Confidence limits für 95% liegen, ist also eine funktionell intakte Erythropoese anzunehmen. Die von uns untersuchten Fälle von hämolytischer Anämie liegen, abgesehen von einer etwas größeren Streuung, ebenfalls in diesem Bereich.

Aus den mitgeteilten Ergebnissen geht bereits der praktische Wert der Methode unter Anwendung dieses Bezugssystems zur Erkennung von hypo- und aplastischen Zustandsbildern der Erythropoese hervor Eine hochgradige Verminderung der Erythropoese findet sich erwartungsgemäß bei Anämien mit leerem oder zellarmem Knochenmark. Bei den untersuchten Fällen von unbehandelter Perniciosa lagen die Werte weit unter der Norm. Dies ist ein Beispiel dafür daß aus den morphologischen Erythropoesezeichen im Knochenmark keine sicheren Schlüsse auf die tatsächliche effektive Erythropoese gezogen werden dürfen. Ähnliche Überlegungen gelten für die Eisenmangelanämie. Eine hochgradige Verminderung der effektiven Erythropoese findet sich weiterhin bei unbe-

handelten akuten Leukosen. In den Anfangsstadien der chronischen lymphatischen Leukämie und des Lymphogranuloms ist die effektive Erythropoese in der Regel normal, erst mit dem Fortschreiten dieser Erkrankungen nimmt sie ab und sinkt schließlich auf sehr niedrige Werte. Aus der wiederholten Bestimmung der Aktivität kann demnach ein weiteres prognostisches Kriterium gewonnen werden.

Von praktischem therapeutischen Interesse erscheint die Bestimmung der G-6-PDH Aktivität der Erythrozyten im Verlaufe der Therapie von malignen Bluterkrankungen. Bei normalen Ausgangswerten muß ein Absinken der Aktivität als Warnzeichen aufgefaßt werden, da die intakte effektive Erythropoese nunmehr geschädigt wird. Ein Absinken der Aktivität bei bereits erniedrigten Ausgangswerten im Verlauf der Therapie ist prognostisch als ungünstig zu bewerten, ein Ansteigen kann die Remission anzeigen.

Ein gutes Beispiel für die Erfassung einer mangelhaften effektiven Erythropoese stellt die Bestimmung der G-6-PDH-Aktivität im Verlaufe von akuten Anurien dar. Während die Fermentaktivität bereits kurz nach Eintreten der akuten Anurie auf extrem niedrige Werte abfällt, tritt die Anämie erst nach zwei bis drei Wochen ein. Umgekehrt läßt sich die Erholung der Erythropoese bei noch anämischen Patienten nach Überwindung der Anurie aus dem Ansteigen der Fermentwerte ablesen.

Der wesentliche neue Gesichtspunkt zur Beurteilung der effektiven Erythropoese bei Vorliegen einer Anämie auf Grund der Bestimmung der G-6-PDH Aktivität in den Erythrozyten liegt darin, daß die gemessenen Werte zum Hämoglobingehalt des Blutes in Bezug gebracht werden.

Zusammenfassung

Die Abnahme der G-6-PDH-Aktivität während der physiologischen Alterung der Erythrozyten wird benützt, um über das mittlere Lebensalter einer Erythrozytenpopulation Aufschluß zu erhalten. Auf die effektive Erythropoese kann unter Berücksichtigung des Hämoglobingehaltes im Vollblut geschlossen werden. Ein geeignetes Bezugsystem zur Beurteilung der effektiven Erythropoese bei verschiedenen Anämien wird aufgestellt. Die Ergebnisse bei verschiedenen Bluterkrankungen und die Beeinflussung der effektiven Erythropoese durch therapeutische Maßnahmen werden mitgeteilt und diskutiert.

Summary

The decrease in glucose-6-phosphate dehydrogenase activity during the process of physiological aging of erythrocytes is used to assess the average age of an erythrocyte population. By taking the hemoglobin content of the whole blood into consideration,

the rate of effective erythropoiesis can be estimated. A suitable reference system is outlined for comparing the effective erythropoiesis in various types of anaemia. The results in various blood dyscrasias and the modification of effective erythropoiesis by treatment are reported and discussed.

Résumé

Les auteurs utilisent la diminution de l'activité de la G-6-PDH au cours du vieillissement physiologique des érythrocytes pour obtenir des indications sur la durée moyenne de la vie des érythrocytes. Il est possible d'apprécier l'érythropoïèse effective sur la base de la concentration de l'hémoglobine dans le sang. Un système de référence approprié pour l'appréciation de l'érythropoïèse effective des diverses anémies est construit. Les auteurs rapportent et discutent les résultats obtenus chez différentes maladies hématologiques et l'influence des mesures thérapeutiques sur l'érythropoïèse effective.

Literatur

1. Butta, F Dittami, M. Marasini, G. Vergnano, F e Gavosto, F Il metabolismo glucidico durante la senescenza eritrocitaria. I. Modificazioni di alcuni enzimi della ta collaterale dell'esosomonofosfato. Arch. Sci. med. *108*: 255–272 (1959).
2. Finch, C. A. and Noyes, W. D. Erythrokinetics in diagnosis of anemia. J. Amer. med. Ass. *175*: 1163–1166 (1961).
3. Gavosto, F ; Butta, F Dittami, M.; Marasini, G. e Vergnano, P Modificazioni delle transaminasi eritrocitarie durante la senescenza in vivo. Arch. Sci. med. *108*: 377–390 (1959).
4. Giammanco, L. Rapporti tra attività della glucosio-6-fosfato-deidrogenasi, concentrazione del glutatione ridotto corpuscoli di Heinz negli eritrociti normali. Med. Lavoro *51* 59–67 (1960).
5. Levy L. M. Walter, H. and Sass, M. D. Enzymes and radioactivity in erythrocytes of different ages. Nature *184* 643–644 (1959).
6. Löhr, G. W und Waller, H. D. Zellstoffwechsel und Zellalterung Klin. Wschr. *37* 833–843 (1959).
7. Löhr, G. W und Waller, H. D Zur Biochemie der Erythrocytenalterung. Folia haemat., Lpz. *78*. 385–398 (1962).
8. Löhr, G. W Waller, H. D Karges, O Schlegel, B. und Müller, A. A. Zur Biochemie der Alterung menschlicher Erythrocyten. Klin. Wschr. *36*. 1008–1013 (1958).
9. Marks, P A. A relationship between human erythrocyte aging in vivo and the activities of glucose-6-phosphate and 6-phosphogluconic dehydrogenases. J. clin. Invest. *36*. 913 (1957).
10. Marks, P A. Red cell glucose-6-phosphate and 6-phosphogluconic dehydrogenases and nucleoside phosphorylase. Science *127* 1338–1339 (1958).
11. Marks, P A. Johnson, A. B. Hirschberg, E. and Banks, J Studies on the mechanism of aging of human red blood cells. Ann. N. Y Acad. Sci. *75*. 95–105 (1958).
12. Nicola, E. A. Bestämning av enzymer i erythrocyter Nord. Med. *68*: 1244–1247 (1962).
13. Pritchard, J A. Erythrocyte age and cholinesterase. Amer J. Physiol. *158*: 72–76 (1949).
14. Rubinstein, D Ottolenghi, P and Denstedt O. F Canad. J Biochem. *34* 222 (1956) zit. nach Bauer, K. U Enzymopathologie der Blutzellen. Erg. *6*: 239–252 (1962).

15. Sabine, J. C.: Choline esterase of blood cells and plasma in blood dyscrasias, with special reference to pernicious anemia. J. clin. Invest. *19*: 833–842 (1940).
16. Sabine, J. C. The cholinesterase of erythrocytes in anemias. Blood *6*: 151–159 (1951).
17. Sabine, J. C.: Erythrocyte cholinesterase titers in hematologic disease. Amer. J. med. *27*: 81–86 (1959).
18. Sailer, S., Sandhofer, F. und Braunsteiner, H. Enzymgehalt der Erythrozyten und effektive Erythropoese. Wien. Z. inn. Med. *44*: 31–34 (1963).
19. Sass, M. D. and Spear, P. W. Whole blood transaminase levels in anemia. J. Lab. clin. Med. *51*: 926–934 (1958).
20. Sass, M. D. and Spear, P. W. Red cell transaminase levels in anemia. II. Thalassemia minor. J. Lab. clin. Med. *58*: 580–585 (1961a).
21. Sass, M. D. and Spear, P. W.: Red cell transaminase levels in anemia. III. Acute and chronic blood loss. J. Lab. clin. Med. *58*: 586–591 (1961b).
22. Scudamore, H. H.; Vorhaus, L. J. and Kark, R. M. Observations on erythrocyte and plasma cholinesterase activity in dyscrasias of the blood. Blood *6*: 1260–1273 (1951).
23. Tocantins, L. M.; Crosby, W. H.; Finch, C. A.; Hagman, F. L.; Stohlman, F. Jr. and Wasserman, L. Modern methods for quantitation of red blood cell production and destruction. Erythrokinetics. Blood *18*: 225–235 (1961).

Adresse der Autoren: Dr. S. Sailer, Dr. F. Sandhofer und Doz. Dr. H. Braunsteiner, II. Medizinische Univ.-Klinik, Garnisongasse 13, Wien IX (Österreich).

Table II

Blood smears of parents.

		Hypochromia	Anisocytosis	Poikilocytosis	Microcytosis	Target cells	Basophilic stippling	Ovalocytes	Polychromasia
A. A.	m	mild	+	+	+	+	+	+++	0
	f	mild			+	+	+	++	++
F. A.	m	moderate	++	+++	+	++	+	++++	+
	f	mild	+	+	+	+++	++	+++	++
M. C.	m	moderat	+	++	+	++	0	++++	+
	f	moderate	+	+	+	+	0	++	+
S. C.	m	mild	+	+	+	+	0	++	0
	f	mild	+	+	+	+++	+	++	+
A. C.	m	mild	+	+	+	0	0	++++	0
	f	moderate	+++	++	+	++	+	++++	+
P. C.	m	moderate	++	+	+	++	0	+++	++
	f	moderate	++	++	+	++	++	+++	++
L. J.	m	moderat	+	+	+	+	0	++	+
	f	bsent	+	+	+	+	+	++++	+
P. K.	m	moderate	+	+	+	++	+	+++	+
	f	mild	+	++	+		++		
E. K.	m	moderate	+	+	+	++	+	+++	+
	f	absent	+	+	0	0	+	++	
D. L.	m								
	f	moderate	+	+	+	0	++	+++	+
F. M.	m	moderate	+	+	+	++	0	+++	+
	f	moderate	+	+	+	++	+	++	+
R. M.	m	mild	+	+	+	0	0	++	
	f	mild	+	+	0	+	+	0	
R. S.	m	moderate	+++	+	+	++++	+		
	f	moderate	+	+	+	++	++++		+
F. Z.	m	moderate	+	+	+	++	+	+	
	f	moderate	+	+	+	++	+++		

Legend

+ = 1 or 2 erythrocytes affected in occasional high power fields

++ = 1 or 2 erythrocytes affected in each high power field

+++ = several erythrocytes affected in each high power field

++++ = many erythrocytes affected in each high power field

were performed by the alkali denaturation method (13). Levels of A_2-hemoglobin were determined by starch block electrophoresis* utilizing the technique of KUNKEL AND WALLENIUS (14) as modified by LEHMANN (15). Reticulocyte counts on the patients were performed by the new methylene blue method (16)

Results

Table I shows the A_2-hemoglobin levels and fetal hemoglobin levels of the parents of our subjects. The A_2-hemoglobin was greater

* These studies were performed in part through the courtesy of Dr. HOWARD PEARSON of the Naval Hospital in Bethesda, Maryland, and in part in our own laboratory

Table III

A_2-Hemoglobin and fetal hemoglobin levels of patients.

	A_2-Hemoglobin %	Fetal hemoglobin %
A. A.	1.9	13.0
F. A.	2.1	76.9
M. C.	1.2	63.8
S. C.	3.5	15.0
A. G.	3.3	60.0
P. C.	1.9	62.5
L. J.	1.2	55.0
P. L.	2.9	63.2
E. K.	2.4	85.0
D. L.	2.4	17.9
F. M.	3.0	76.0
R. M.	3.3	65.0
R. B.	1.6	71.8
F. Z.	1.8	64.0
Normal values	3.6 or less	2.0 or less

than the upper limit of normal for our laboratory (3.6 %) in both parents of all but two of the patients. The father of F. A. and the mother of E. K. had normal A_2-hemoglobin levels. The fetal hemoglobin level was greater than the upper limit of normal (2.0 %) in one or both parents of 10 patients. The level was elevated considerably in one parent of each of two of these patients (F. A. and E. K.) In the remainder of the parents the fetal hemoglobin levels were within the normal range.

The blood smears of all the parents were abnormal (table II) with either slight or moderate anisocytosis and poikilocytosis all but two were hypochromic. In some smears, target cells, basophilic stippling, and ovalocytes were present.

The A_2-hemoglobin levels of the patients determined at least one month and usually two months following the last transfusion were within the range of normal in all cases (table III) Fetal hemoglobin levels were markedly elevated in all but three patients. The latter all of whom required transfusions very frequently were tested shortly after a transfusion had been given.

The blood smears of the patients all showed hypochromia, marked anisocytosis, and moderate to marked poikilocytosis (table IV) Five patients had many target cells and 5 had numerous red blood cells with basophilic stippling One patient had many ovalocytes, while 7 others showed only a moderate number Poly

Table II
Blood smears of parents.

		Hypochromia	Anisocytosis	Poikilocytosis	Microcytosis	Target cells	Basophilic stippling	Ovalocytes	Polychromasia
A. A.	m	mild	+	+	+	+	+	+++	0
	f	mild			+	+	+	++	++
F. A.	m	moderat	++	+++	+	++	+	++++	+
	f	mild	+	+	+	+++	++	+++	+
M. C.	m	moderate	+	++	+	++	0	++++	+
	f	moderat	+	+	+	+	0	++	[illegible]
S. C.	m	mild	+	+	+	+	0	++	
	f	mild	+	+	+	+++	+	++	
A. C.	m	mild	+	+	+	0	0	++++	
	f	moderate	+++	++	+	++	+	++++	
P. C.	m	moderat	++	+	+	++	0	+++	
	f	moderat	++	++	+	++	++	+++	
I. J.	m	moderate	+	+	+	+	0	++	
	f	absent	+	+	+	+	+	++++	
P. K.	m	moderate	+	+	+	++	+	+++	
	f	mild	+	++	+		++		
R. K.	m	moderat	+	+	+	++	+	+++	
	f	absent	+	+	0	0	+	++	
D. L.	m								
	f	moderate	+	+	+	0	++	+++	
F. M.	m	moderate	+	+	+	++	0	+++	
	f	moderate	+	+	+	++	+	++	
R. M.	m	mild	+	+	+	0	0	++	
	F	mild	+	+	0	+	+	0	
R. S.	m	moderate	+++	+	+	++++	+		
	f	moderate	+	+	+	++	++++		
F. Z.	m	moderate	+	+	+	++	+	+	
	f	moderate	+	+	+	++	+++		

Legend
+ = 1 or 2 erythrocytes affected in occasional high power fields
++ = 1 or 2 erythrocytes affected in each high power field
+++ = several erythrocytes affected in each high power field
++++ = many erythrocytes affected in each high power field

were performed by the alkali denaturation method (13). Levels of A_2-hemoglobin were determined by starch block electrophoresis utilizing the technique of KUNKEL AND WALLENIUS (14) as modified by LEHMAN (15). Reticulocyte counts on the patients were performed by the new methylene blue method (16).

Results

Table I shows the A_2-hemoglobin levels and fetal hemoglobin levels of the parents of our subjects. The A_2 hemoglobin was greater

---

These studies were performed in part through the courtesy of Dr. HOWARD PEARSON of the Naval Hospital in Bethesda, Maryland, and in part in our own laboratory.

Table III

A_2-Hemoglobin and fetal hemoglobin levels of patients.

	A_2-Hemoglobin %	Fetal hemoglobin %
A. A.	1.9	13.0
F. A.	2.1	76.9
M. C.	1.2	63.8
S. C.	3.5	15.0
A. C.	3.3	60.0
P. C.	1.9	62.5
L. J.	1.2	55.0
P. K.	2.9	65.2
E. K.	2.4	85.0
D. L.	2.4	17.9
F. M.	3.0	76.0
R. M.	3.3	65.0
R. S.	1.6	71.8
F. Z.	1.8	64.0
Normal values	3.6 or less	2.0 or less

than the upper limit of normal for our laboratory (3.6 %) in both parents of all but two of the patients. The father of F. A. and the mother of E. K. had normal A_2-hemoglobin levels. The fetal hemoglobin level was greater than the upper limit of normal (2.0 %) in one or both parents of 10 patients. The level was elevated considerably in one parent of each of two of these patients (F. A. and E. K.). In the remainder of the parents the fetal hemoglobin levels were within the normal range.

The blood smears of all the parents were abnormal (table II) with either slight or moderate anisocytosis and poikilocytosis; all but two were hypochromic. In some smears, target cells, basophilic stippling, and ovalocytes were present.

The A_2-hemoglobin levels of the patients determined at least one month and usually two months following the last transfusion were within the range of normal in all cases (table III). Fetal hemoglobin levels were markedly elevated in all but three patients. The latter, all of whom required transfusions very frequently, were tested shortly after a transfusion had been given.

The blood smears of the patients all showed hypochromia, marked anisocytosis, and moderate to marked poikilocytosis (table IV). Five patients had many target cells and 5 had numerous red blood cells with basophilic stippling. One patient had many ovalocytes, while 7 others showed only a moderate number. Poly-

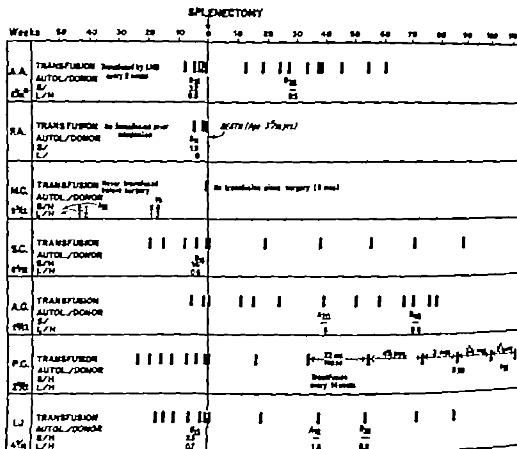


Fig 1A

Fig 1 Transfusion requirements, erythrocyte survival studies, and sites of erythrocyte sequestration.

A or Autol	chromated autologous erythrocytes	S/H	spleen/heart radioactivity
D or Donor	chromated donor erythrocytes	L/H	liver/heart radioactivity

Numbers listed below initials refer to age in years at time of splenectomy

chromasia was noted in all but one patient and was marked in 7 Nucleated erythrocytes were present in the smears of all but one subject and markedly increased in 8.

Transfusion requirements and erythrocyte survival studies are shown in fig 1

A Presplenectomy Transfusion requirements of the patients showed intervals ranging from 2 weeks to 6 months during the period studied the shortest intervals were found just prior to splenectomy Hemoglobin levels at the time of transfusion ranged

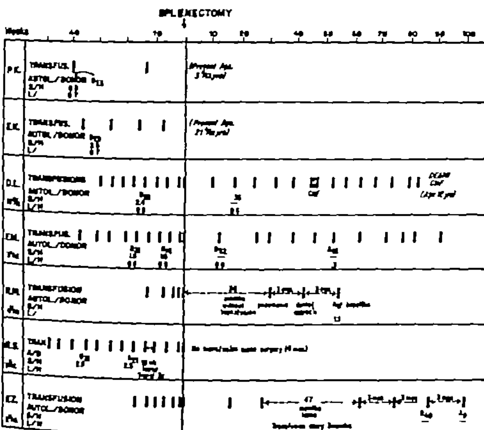


Fig 1B

from 1 6 gm / to 7.9 gm % with a median of 5.2 gm %. Reticulocyte levels determined on 6 of the patients during the presplenectomy period ranged from 0 1 / to 7.6 / with a median of 3.7 %. Autologous erythrocyte survival studies performed in 2 patients (R. S. and M. C.) showed a shortened Cr^{51}-erythrocyte half-life of 21 days in both (normal 28 to 40 days) The latter patient (M. C.) was restudied 18 months later and the half life was found to have decreased slightly This patient had never been transfused. The half-life of donor erythrocytes was determined in 9 patients and found to range from 11 to 22 days in 8 in one patient it was 29 days.

In the patients studied before splenectomy autologous erythrocytes were sequestered primarily in the spleen although some uptake was also detectable in the liver Donor erythrocytes were sequestered

Table IV
Blood smears of patients.

	Hypochromia	Anisocytosis	Poikilocytosis	Microcytosis	Target cells	Basophilic stippling	Ovalocytes	Polychromasia	Nucleated RBC
A. A.	moderate	++++	+++	+	++	++++	+	++++	++
F. A.	moderate	+++	++++	+	+	++		++++	+
M. C.	moderate	+++	+++	+	+	+++	++	++++	[illegible]
S. C.	moderate	++++	+++	+	++++	++	++	+++	+++
A. C.	moderate	++++	+++	+	+++	++++	++++	+++	+++
P. C.	moderate	++++	++	+	+++	++	0	+++	+++
L. J.	moderate	++++	+++	+	++++	++++	+++	++++	++++
P. K.	moderate	++++	++++	+	++	++		++++	++++
E. K.	moderate	++++	+++	+	+	++++	++	+	+
D. L.	moderate	++++	++	+	++++	+	0	+++	+++
F. M.	moderate	++++	+++	+	++++	+	+++	0	+++
R. M.	moderate	++++	+++	0	++++	+	0	+++	+++
R. S.	moderate	++++	++++	+	++	++++		++++	++++
F. Z.	moderate	+++	++	+	+++	++	++	++++	++++

Legend

\+ = 1 or 2 erythrocytes affected in occasional high power fields
++ = 1 or 2 erythrocytes affected in each high power field
+++ = several erythrocytes affected in each high power field
++++ = many erythrocytes affected in each high power field

mainly in the spleen. Only one patient had no increase of splenic uptake of donor cell radioactivity She was 2½ years old (the youngest one in the group) had only minimal clinical splenomegaly and had not required transfusions prior to the date of the study

B Postsplenectomy The transfusion intervals of the splenectomized patients at the time erythrocyte survival studies were performed ranged from 4 weeks to 4 months with 3 exceptions. The exceptions included one patient (R. M.) who was transfused only 3 times in the 4 years following splenectomy and 2 patients (R. S. and M. C.) who had not been transfused following splenectomy 6 months and 9 months ago, respectively At the time transfusion was given hemoglobin levels ranged from 3.6 gm% to 10.7 gm% with a median of 6.2 gm%. Reticulocyte levels ranged from less than 0.1% to 11.7% with a median of 3.8%.

The half-life of autologous erythrocytes was determined in 6 patients and showed significant shortening with a range of 9–21 days. The donor erythrocyte half-life was studied in 7 subjects and was found to be within the normal range of 28–40 days in all. After splenectomy some sequestration of autologous erythrocytes in

the liver was observed in all 6 patients. Donor erythrocytes, however were not sequestered in the liver

Discussion

A number of investigators have studied erythrocyte production and destruction in patients with thalassemia major STURGEON AND FINCH (17) employing Fe^{59} and Cr^{51} techniques reported an increased rate of erythrocyte destruction and a hemoglobin turnover comparable to that of other severe anemias however they observed a defect in effective erythrocyte production. The half life of autologous erythrocytes in 3 patients was 7 9 and 10 days. Splenectomy did not seem to affect the rate of erythrocyte production. ERLANDSON et al. (2) utilized the Cr^{51} technique to study 10 patients 6 with "severe disease" had the most marked hemolytic defect. From calculations of hemoglobin and erythrocyte production, they concluded that the degree of anemia could not be correlated solely with the rate of erythrocyte destruction or of erythrocyte production or type of hemoglobin produced (adult or fetal) No patient was studied both before and after splenectomy It is noteworthy that one patient who had been splenectomized and had shown subsequent clinical improvement nevertheless had one of the highest rates of autologous erythrocyte destruction observed. GRINSTEIN et al (18) employing radioactive glycine, showed that the incorporation of glycine into the hemoglobin of patients with thalassemia major was delayed and decreased, whereas labeling of stercobilin was accelerated and increased. This was interpreted to mean that delivery of the erythrocytes was ineffective and that stercobilin formation increased as a result of abortive heme synthesis. These data, and the observations of STURGEON AND FINCH (17) have recently been confirmed by HAURANI AND TOCANTINS (19) using both the Fe^{59} and Cr^{51} techniques, and have been reviewed and discussed by BANNERMAN (20)

In the present study all the patients appear to have thalassemia major The parents of all but two patients conform to the strict criteria of GERALD AND DIAMOND (21) for the diagnosis of thalassemia minor

In presplenectomy patients there was no correlation between reticulocyte counts and the need for transfusion except in one subject, who had persistent reticulocytopenia and required the greatest

number of transfusions. In this patient no conclusion can be drawn regarding impaired hemoglobin or erythrocyte production because frequent transfusions may have resulted in secondary depression of erythropoiesis. Postsplenectomy patients with nearly identical reticulocyte counts had widely varying transfusion requirements. The median reticulocyte counts obtained before splenectomy did not differ from those in postsplenectomy patients. Data concerning autologous erythrocyte life-span prior to splenectomy cannot be evaluated because studies were performed on only 2 subjects. The shortened donor erythrocyte survival time previously reported for patients with thalassemia major before splenectomy was found in all but one of the subjects in this study There did not seem to be a direct relationship between this and the transfusion requirement. It is possible that if serial studies were carried out on individual subjects, a closer correlation might be found for donor erythrocyte survival and transfusion requirement.

All postsplenectomy patients showed a shortening of the autologous erythrocyte life-span, but no correlation was found between the life-span and transfusion intervals. For example, the subject R. M. who showed the least need for transfusion, had an autologous erythrocyte half life of 17 days, shorter than those of several other patients who required more frequent transfusions. The donor erythrocyte life-spans were normal in all patients following splenectomy in agreement with the findings of other investigators. They have been observed by us to remain normal for as long as 5 years after splenectomy (F Z.) Although the transfusion requirement was decreased in all subjects in the postsplenectomy period, the interval between transfusions varied greatly

Presplenectomy the site of autologous erythrocyte sequestration appears to be the spleen and to a lesser extent other reticuloendothelial areas, as represented by the liver Sequestration of donor cells occurs almost exclusively in the spleen with little or none in other reticuloendothelial areas. The one patient (P K.) whose splenic sequestration of donor erythrocytes was normal is the youngest in our group and had minimal splenic enlargement at the time of study Although no conclusive statement regarding the correlation between splenic sequestration of donor erythrocytes and transfusion interval can be made, it is noteworthy that the patient (P K.) who had a normal spleen/heart ratio (S/H) required no transfusions, whereas the patient (A. A.) with the highest S/H

ratio had the greatest transfusion need. Postsplenectomy the sequestration of autologous erythrocytes occurred in the liver and probably in other reticuloendothelial areas. Donor erythrocyte sequestration remained entirely normal. Although it is possible that other sites of erythrocyte destruction develop with time, our data concerning the uptake of radioactivity by the liver do not prove this. It would seem that factors other than erythrocyte destruction by the spleen or other reticuloendothelial areas determine the hemoglobin level in these patients.

It is our conclusion that none of the parameters studied to date either by others or in the present report can be used as a sole index for determining the present need for blood transfusion or the future course of the disease in thalassemia major

Summary

Clinical status, transfusion requirements, certain laboratory findings, and the survival and sites of destruction of autologous and donor erythrocytes have been evaluated and compared in 14 subjects with thalassemia major. Although varying requirements for transfusion were recognized both before and after splenectomy in different patients of this series, the need for blood transfusion was not closely correlated with hereditary aspects, erythrocyte morphology reticulocyte levels, or the survival and sites of destruction of either autologous or donor erythrocytes. In subjects with thalassemia major no single parameter alone may be relied upon to determine management.

Résumé

Les auteurs étudient et comparent l'évolution clinique, le nombre de transfusions nécessaires, certains résultats de laboratoire ainsi que la survie et les lieux de destruction des érythrocytes autologues ou provenant d'un donneur chez 14 malades, souffrant d'une thalassémie majeure. Bienque le nombre de transfusions nécessaires avant et après la splénectomie différait chez plusieurs malades, les auteurs ne pouvaient trouver une relation entre la nécessité d'une transfusion et les caractères héréditaires, la morphologie des érythrocytes, le nombre réticulocytaires, la morphologie des érythrocytes, le nombre réticulocytaire, la survie ou les lieux de destruction des érythrocytes autologues ou transfusés. Il n'est pas possible de trouver un seul critère valable chez les thalassémiques, qui, apprécié tout seul, est significatif pour le traitement.

Zusammenfassung

Bei 14 Patienten mit Thalassaemia major wurden das klinische Bild, der Bedarf an Transfusionen, gewisse Laboratoriumsbefunde, sowie die Überlebenszeit und die Destruktionsstätten von autologen und von Spendererythrozyten untersucht und verglichen. Obgleich sich bei verschiedenen Patienten dieser Serie sowohl vor als auch nach Splenektomie ein verschiedener Bedarf an Transfusionen ergab, ließ sich die Notwendigkeit zur Vornahme von Bluttransfusionen nicht in Beziehung bringen mit erblichen Merkmalen, Erythrozytenmorphologie, Retikulozytenzahl oder Überlebensdauer und Destruktionsstätten autologer oder gespendeter Erythrozyten. Bei Patienten mit Thalassaemia major läßt sich kein einzelnes Kriterium aufstellen, das für sich allein für die Behandlung maßgebend ist.

References

1. Bailey, J. S. and Prankerd, T. A. J. Studies in thalassemia. Brit. J. Haemat. 4: 150 (1958).
2. Erlandson, M. E. Schulman, I. Stern, G. and Smith, C. H. Studies of congenital hemolytic syndromes. I. Rates of destruction and production of erythrocytes in thalassemia. Pediatrics 22: 910 (1958).
3. Hamilton, H. E. Sheets, R. F and DeGowin, E. L. Studies with inagglutinable erythrocyte counts. II. Analysis of the mechanism of Cooley's anemia. J. clin. Invest. 29: 714 (1950).
4. Kaplan, E. and Zuelzer, W. W. Erythrocyte survival studies in childhood. II. Studies in Mediterranean anemia. J. Lab. clin. Med. 36: 517 (1950).
5. Smith, C. H. Erlandson, M. E. Stern, G. and Schulman, I. The role of splenectomy in the management of thalassemia. Blood 15: 197 (1960).
6. Lichtman, H. C. Watson, R. J. Feldman, F; Ginsberg, V and Robinson, J. Studies on thalassemia. Part I. An extra corpuscular defect in thalassemia major. Part II. The effects of splenectomy in thalassemia major with an associated acquired hemolytic anemia. J. clin. Invest. 32: 1229 (1953).
7. Smith, C. H. Schulman, I. Ando, R. E. and Stern, G. Studies in Mediterranean (Cooley's) anemia. I. Clinical and hematologic aspects of splenectomy with special reference to fetal hemoglobin synthesis. Blood 10: 582 (1955).
8. Clement, D. H. and Taffel, M. Splenectomy in Mediterranean anemia. Pediatrics 16: 353 (1955).
9. Reemtsma, K. and Elliott, R. H. E. Jr. Splenectomy in Mediterranean anemia: an evaluation of long-term results. Ann. Surg. 144: 999 (1956).
10. Wolff, J. A. Sitarz, A. L. and Von Hofe, F. H. Effect of splenectomy on thalassemia. Pediatrics 26: 674 (1960).
11. Madden, R. A. and O'Connor, W. J. Evaluation of splenectomy in the treatment of Cooley's anemia. Ann. Surg. 148: 44 (1958).
12. Ultmann, J. E. The role of the spleen in the hemolytic anemia of cancer patients. Cancer Res. 18: 959 (1958).
13. Singer, K. Chernoff, A. I. and Singer, L. Studies on abnormal hemoglobins. I. Their demonstration in sickle cell anemia and other hematologic disorders by means of alkali denaturation. Blood 6: 413 (1951).
14. Kunkel, A. G. and Wallenius, G. New hemoglobin in normal adult blood. Science 122: 288 (1955).
15. McCoo, J. W. Jr. and Lenner, S. A rapid modified starch electrophoretic method. Amer. J. clin. Path. 35: 349 (1961).
16. Brecher, G. New methylene blue as a reticulocyte stain. Amer. J. clin. Path. 19: 895 (1949).
17. Sturgeon, P. and Finch, C. A. Erythrokinetics in Cooley's anemia. Blood 12: 64 (1957).
18. Grinstein, M.; Bannerman, R. M. Vavra, J. D. and Moore, C. V. Hemoglobin metabolism in thalassemia. In vivo studies. Amer. J. Med. 29: 18 (1960).
19. Haurani, F. I. and Tocantins, L. M. Ineffective erythropoiesis. Amer. J. Med. 31: 519 (1961).
20. Bannerman, R. M. Thalassemia, A Survey of Some Aspects; in Modern Medical Monographs 23 (Grune and Stratton, New York/London 1961).
21. Gerald, P. S. and Diamond, L. K. The diagnosis of thalassemia trait by starch block electrophoresis of the hemoglobin. Blood 13: 61 (1958).

Authors' address: Drs. Anneliese L. Sitarz, J. E. Ultmann and J. A. Wolff, The College of Physicians and Surgeons, 630 West 168th Street, New York City 32, N.Y. (USA).

Acta haemat. 30 215-220 (1963)

2nd Internal Clinic of Istanbul Medica[illegible] Hasek[illegible] Capa, Istanbul

The Combination of Hered[illegible] [illegible]ptocytosis with Heterozygous Be[illegible] [illegible]aemia*

Study of [illegible] T[illegible]

By MUZAF[illegible]

The combination of heredit[illegible] [illegible] with other hereditary blood dyscrasies are theo[illegible] But up to now only a few instances of hereditar[illegible] [illegible]ociated with other traits have been identified. [illegible] [illegible]ditary elliptocytosis associated with sickle cell trait [illegible] [illegible]ygous haemoglobin S disease plus hereditary elliptocytosis [illegible] the combination of hereditary elliptocytosis with haemoglobin C [illegible] 2) and hereditary elliptocytosis plus heterozygous thalassaemia (3, 4).

Recently we have examined the family members of a patient with COOLEY's anaemia. The patient's mother had haematologic findings of beta thalassaemia and also morphologic characteristic of hereditary elliptocytosis. Therefor[illegible] we strongly suspected that the patient's mother was simultaneously carrying a gene for beta thalassaemia and also a gene responsible for hereditary elliptocytosis. The genetic study of the mother confirmed this possibility.

The purpose of this paper is to report a family with the combination of hereditary elliptocytosis with beta thalassaemia and to discuss the difference in the clinical and haematologic picture between this patient and the other instances described previously.

Methods

The haematologic methods were described in other articles (5, 6). Haemoglobin A_2 was determined by the method of discontinuous TRIS buffer paper electrophoresis according to the modified technique of GOLDBERG (7, 8) also by starch gel electrophoresis according to the method of SMITHIES (9). The maximum normal values for haemoglobin A_2 in these methods were 3.5% for the former and 5% for the latter.

* This investigation was supported by [illegible] grant from the Blood Research Foundation, Washington, D. C.

Table I

Haematologic data on family with the combination of hereditary elliptocytosis and heterozygous beta-thalassaemia.

	Case 2 Mother	Case 1 Patient	Case 3 Father	Case 5 Father of Case 2	Case 4 Mother of Case 2
RBC 10^6/cu.mm.	4.80	3.10	5.20	4.60	6.00
Haemoglobin, g	11.2	7.2	12	12	12.8
Color index	0.7	0.77	0.78	0.89	0.73
WBC/mm³	7.200	23.000	6.700		
Retics.,	2	14.8	1		
Haematocrit, %	38	23	45	42	48
MCV μ^3	79	74	86	86.9	80
MCHb., $\gamma\gamma$	23.3	23.2	23	26	21.3
MCHb.C, %	29.4	31	26.6	28.5	26.6
NRC/100 WBC	0	31	0	0	0
Anisocytosis	+++	++++	++	—	++
Polychromasia	—	++	—	—	—
Poikilocytosis	+	++++	++	—	+
Microcytosis	++	++++	++	—	+
Hypochromea	++	+++	++	—	+
Ovalocytes, %	26.6	10.4	8	21.4	18
Elliptocytes, %	14.4	0.6	0	2	0
Target-cells	+	++	+	—	+
Osmotic fragility % NaCl	0.38–0.28	0.38–0.22	0.42–0.32	—	—
Total bilirubin, mg%	1	3.2	0.8		
Serum iron, γ%	200	60	100		
Fetal haemoglobin,	0	63	0	0	0
Haemoglobin A_2,	3.7	2.5	4	3.3	4.4
Haemoglobin pattern	A	AF	A	A	A

The low serum iron possibly is due to the presence of an infection when this determination was performed.

Haemoglobin A_2 contents are determined by the method of discontinuous TRIS buffer paper electrophoresis (8). The results of case 2 and 3 were controlled by Drs. K. Betke and Kleihauer, Tübingen, Germany by starch block electrophoresis.

Family Report

Case 1 (J. A.) A 2½ year old girl from Izmir came to the outpatient department of the 2nd Internal Clinic of Istanbul Medical School because of anaemia, jaundice lassitude and fever. According to her parents statement she had been anaemic since early infancy. She was diagnosed as Cooley' anaemia in one hospital in Izmir. She was very pale looking girl. She had mongoloid facies and striking frontal bossing. Her sclerae were subicteric. A loud systolic murmur was heard over entire praecordium. The spleen extended three fingerbreadths and the liver one fingerbreadth below the costal margin. Her haematologic findings were consistent with the diagnosis of Cooley anaemia and summarized in table I. X ray of the skull exhibited marked thickening of the diploe and radial striation. A bone survey demonstrated marked osteoporosis with honey-combed" appearance around the elbow joint and there was striking osteoporosis of the bones of the hands resulting in "mosaic pattern.

Case 2, the mother of case 1 (S. A.) A 29 year old Turkish woman from Izmir was examined during the family study of her daughter J. A. She was entirely asymptomatic and apparently healthy. Her haematologic data and genetic study (fig. 1) showed

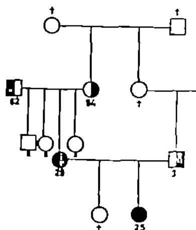


Fig 1 Genealogy of Turkish family w ... heterozygous beta-thalassemia (numbers ...

the combination of hereditary elliptocytosis ... thalassaemia. Her laboratory data are summarized in table I.

Case 3, the father of case 1 (U A.) ... man from Izmir the first maternal cousin of his wife. He was entirely normal and healthy. His haematologic data showed that he had heterozygous beta-thalassaemia. His laboratory data are summarized in table I.

Case 4 the maternal grandmother of case 1, mother of case 2 (Z. M.) A 54 year old Turkish woman from Cyprus. Her haematologic data were consistent with the diagnosis of heterozygous beta-thalassaemia. Her laboratory data are summarized in table I.

Case 5, the maternal grandfather of case 1 father of case 2 (A. M.) A 62 year old Turkish man from Cyprus. His blood samples were sent to our laboratory for examination. For this reason, some of the other tests were not performed. He showed morphologic characteristic of hereditary elliptocytosis. His haematologic data are summarized in table I.

Case 6, the paternal grandfather of case 1 father of case 3 (A. M.) A 69 year old man from Macedonia. His blood samples were sent to our laboratory for examination. His haematologic findings were within normal limits. Therefore, his haematologic data are not included in table I.

Comment

If we analyse the haematologic findings and the results of the genetic study we suppose that the mother of our patient (case 2) has the combination of hereditary elliptocytosis with heterozygous beta-thalassaemia. The findings confirming this possibility can be divided into two groups

I The findings showing the presence of a gene responsible for beta-thalassaemia are as follows decreased MCV low MCHb, the presence of target cells, microcytosis and poikilocytosis in the blood smear decreased osmotic fragility increased serum iron and in-

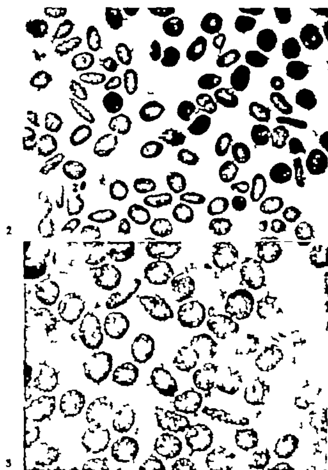


Fig 2 Photomicrograph of the blood film of case 1 showing elliptocytosis, ovalolocytosis, microcytosis and poikilocytosis.

Fig 3 Photomicrograph of the blood film of case 5, father (A. M.) showing elliptocytosis and ovalocytosis.

creased haemoglobin A_2-fraction. These findings are consistent with the presence of a gene responsible for beta-thalassaemia.

2 The findings showing the presence of a gene responsible for hereditary elliptocytosis in case 2 are as follows there was a marked elliptocytosis in the blood smear (fig 2) As it is known thalassaemic syndromes and heterozygous thalassaemia may be associated with marked elliptocytosis. Therefore the very striking elliptocytosis in the propositus can be explained by heterozygous beta-thalassaemia. The only evidence confirming the presence of a gene for hereditary elliptocytosis in the propositus is the result of genetic study (fig 1)

Although one of the parents (the r ... rn. grandm the: ... M had mild stigmata of heterozyg... ... stszemia n ... in creased haemoglobin A_2, the oth ... maternal gr ... the A. M.) did not exhibit the find... thal ssaem ... the other hand, he had the morph ... for hereditary elliptocytosis (fig. 3 ... the results of the genetic study we are ... striking elliptocytosis observed in the pr ... responsible for hereditary elliptocytos...

As can be seen from case ... offspring (case 1) has all clinical manife... findings of COOLEY's anaemia. The str... ... in the mother (case 2) and his father ... the blood smear of the offspring. Therefor... ... the offspring (case 1) has homozygou... heterozygous elliptocytosis.

As already indicated, the mothe... ...tirely asymptomatic, had no anaemia and did not ... for increased haemolysis. She was diagnosed during ... of her daughter with COOLEY's anaemia. Therefore, we assume that the presence of a gene responsible for hereditary elliptocytosis in our case does not appear to potentiate the pathogenicity of the beta thalassaemia gene. According to the results obtained in our case it seems probable that the presence of one gene for beta thalassaemia and one gene for hereditary elliptocytosis in the same individual does not result in a form of congenital haemolytic anaemia caused by the interaction of these two genes. This observation is similar to those of VANDEPITTE AND LOUIS (1) and also of AVERY (2) in individuals presenting combinations of hereditary ellitocytosis with haemoglobins S and C. This fact led VANDEPITTE AND LOUIS to suppose that the haemoglobin S trait does not accentuate the clinical effects of a single gene for hereditary elliptocytosis. Nearly the same fact was observed by AVERY in two negro children who exhibited simultaneously both hereditary elliptocytosis and haemoglobin C trait.

On the other hand, two instances with the combination of hereditary elliptocytosis plus heterozygous thalassaemia described previously (3, 4) were not entirely asymptomatic. The patient described by BRUMPT et al. (3) had rheumatoid arthralgia. The patient described by DE VRIES et al. (4) exhibited marked signs of increased haemolysis.

The difference in the clinical and haematologic picture found in our patient and the other two individuals with hereditary elliptocytosis plus heterozygous thalassaemia may be explained by the great variability *in the expression of heterozygous thalassaemia* in each case respectively

Summary

A patient presenting combination of hereditary elliptocytosis with heterozygous beta-thalassaemia is described. The genetic study showed that the mother had heterozygous beta-thalassaemia and the father had hereditary elliptocytosis. The patient was entirely asymptomatic. The difference in the clinical and haematologic picture between our case and the other two instances described previously is emphasized.

Résumé

Observation d une malade présentant une elliptocytose héréditaire associée à une β-thalassémie hétérozygote. L étude génétique démontra chez la mère une β-thalassémie hétérozygote et chez le père une elliptocytose héréditaire. La patiente ne présentait aucun symptôme clinique. La différence entre l aspect clinique et hématologique de ce cas et de deux autres cas précédents est soulignée.

Zusammenfassung

Es wird über eine Patientin berichtet, die eine Kombination von hereditärer Elliptocytose mit einer heterozygoten Beta-Thalassämie aufwies. Die erbbiologische Untersuchung ergab bei der Mutter eine heterozygote Beta-Thalassämie und beim Vater eine hereditäre Elliptocytose. Die Patientin zeigte keine krankhaften Symptome. Es wird auf die Unterschiede des klinischen und hämatologischen Bildes zwischen diesem Fall und den früher beschriebenen Fällen hingewiesen.

References

1 Vandepitte, J. et Louis, L. L association elliptocytose-drepanocytose. Etude de deux familles. Rev Hémat. *10*: 19 (1955).
2. Avery M. E. Hereditary elliptocytosis and hemoglobin C trait. A report of two cases. Bull. Johns Hopk. Hosp. *98*. 184 (1956)
3. [illegible] L. C. Delabarre, L. C. et Traverse, P M. Double hétérozygotie entre thalassémie et elliptocytose. Proc. VII. Congr Int. Soc. Hemat., 2nd vol. (Pensiero Scientifico Publishers, Roma 1958)
4. de Vries, S. A.; de Jong, J et Frenkel, M. Anémie elliptocytaire hémolytique. Schweiz. med. Wschr *89*: 1078 (1959)
5. Aksoy M. [illegible], A. and Altöprak, H. Thalassaemia syndromes. I. Thalassaemia minor with large amount of fetal haemoglobin. Acta haemat. 25. 136 (1961).
6. Aksoy M. A brief report on the use of starch gel electrophoresis in haemoglobinopathies including sickle cell-haemoglobin E disease and thalassaemia-haemoglobin E disease. New Istanbul Contrib. clin. Sci. *5*. 101 (1962)
7 Goldberg, C. A. A discontinuous buffer system for paper electrophoresis of human haemoglobins. Clin. Chem. *5*. 446 (1959)
8. Van Zanen, G. E. Expression of the haemoglobin S gene on the island of Curaçao (Drukkerij J Ruysendaal, Amsterdam 1962)
9. Smithies, O. Zone electrophoresis in starch gel. Biochem. J *61* 629 (1955).

Author address: Dr. M. Aksoy 2nd Internal Clinic, Istanbul Med. School, Gureba Hospital, Çapa, *Istanbul* (Turkey)

Acta haemat. 30 221–228 (1963)

Studies of Basophils

Variations with Age and Sex*

(With Plate I)

By Ernst Thonnard-Neumann Washington, D. C.

Since the introduction of the direct chamber count of basophilic leukocytes by Moore and James in 1953 (1) several observations have been published on changes in the number of circulating basophils under physiologic conditions (2). However little information has been available until now on spontaneous variations in normal basophil morphology. Arneth in 1921 (3) applied to basophils his classification of neutrophilic leukocytes based on nuclear morphology and found that among these cells also "herrscht die schönste Ordnung". Due to the small number of human basophils and difficulties in identifying the nucleus under the granules Arneth's method did not find any application. Jucker in 1943 (4) reported that the morphology of rabbit basophils was too varied for classification.

In previous work (5) with normal human subjects fluctuations of basophil numbers were found to be preceded, accompanied and occasionally replaced by changes in the position of the granules in the cytoplasm. These changes were correlated with alternating phases of physiologic activity (menstrual cycle) and it was concluded that the various cell types represented basophils of different age. Cells in which the granules had a peripheral distribution were interpreted as the youngest of circulating basophils, those forms in which some of the granules traversed the center of the cell between the lobes of the usually bilobed nucleus represented an intermediate age group and basophils in which the granules covered the cell surface and thereby partially or completely obscured the nucleus were considered as the most mature. In the present study this classification was extended to rabbit basophils and evidence is sub-

* This work was supported by Public Health Service Grant HE 07362 National Institutes of Health, Bethesda, Maryland (USA)

mitted on the basis of basophil numbers and of cell age differentiation through basophil morphology that in man and rabbit a sex linked difference in basophil activity exists.

Materials and Methods

Samples of capillary blood from 299 male and 595 female adult humans and venous blood from 13 male and 32 female rabbits were used. Total basophil numbers were counted in Speirs-Levy counting chambers. For human blood INAGAKI diluting fluid (6) was used and for rabbit blood solution of the following composition 0.05% aqueous solution of toluidine blue in 1.5% acetic acid. Basophil morphology was studied in smears stained with WRIGHT's, PAPPENHEIM' or UNDRITZ toluidine blue stain. Human and rabbit basophils were classified according to the position of the granules as P" (peripheral) I" (intermediate) and U" (uniform) cell types. The term statistical significance refers to the 95% level of confidence.

In the chamber counts Inagaki' fluid permitted good identification of human basophils, the diluting fluid developed by us gave better differentiation of rabbit basophils. Satisfactory staining of human and rabbit basophils in the blood smear was obtained with the Wright stain. Comparative counts of basophils in smears stained with one of the three stains gave almost identical results.

Results

Basophil Numbers

a) Human subjects In two populations consisting of 311 young adults and 193 old people simple white cell and basophil counts were made. The young subjects were healthy white male and female undergraduate college students with an average age of 18 years, the geriatric subjects were over sixty five years old predominantly white men and women without overt signs of chronic and no acute illnesses. In mean numbers of basophils per mm³ blood and per hundred white cells student females exceeded student males, student females exceeded geriatric females, geriatric males exceeded geriatric females and geriatric males did not differ from student males.

In mean numbers of white cells per mm³ student females exceeded student males, geriatric females exceeded student females, geriatric males exceeded student males and geriatric males did not differ from geriatric females.

The differences were statistically significant the tests used were standard t tests The results are summarized in table I

b) Rabbits In 5 male and 6 female adult rabbits a total of 493 white cell and basophil counts were made. The mean counts for each rabbit were computed and these means were used to obtain the group means which were tested Female adult rabbits

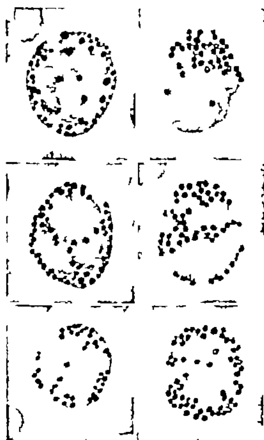


Fig. 1 Basophil morphology and cell age. Left row human basophils, right row rabbit basophils. From top to bottom juvenile ("P" intermediate ("I") and mature ("U") cell types.

Table I

Means of white cells and basophil numbers in .04 human subjects compared by age and sex.

	[illegible]		[illegible] Subjects	
	Males	Females	Males	Females
Numbers of subjects	112	199	97	96
Mean no of WBC per mm³	6,2.0	6,613	,218	7 123
Coefficient of variation, %	19.0	26.2	35.2	31.0
Mean no of basophils per mm³	24 1	27.2	24.1	20.6
Coefficient of variation, %	41.2	45.2	44 1	50.8
Mean percentage of basophils	0.38	0.42	0.37	0.31
Coefficient of variation,	4.0	4 4	4.7	5.0

Coefficient of variation is $\frac{\text{standard deviation} \times 100}{\text{mean}}$

exceeded male adults in the mean numbers of basophils per mm³ and per 100 white cells. Due to wide variations in the individual counts no statistical significance could be attached to these differ ences.

In 11 mature and 12 immature rabbits the mean numbers of basophils were computed in a total number of 756 counts. In mean numbers of basophils mature female rabbits exceeded immature females, mature males did not differ from immature males and immature females did not differ from immature males. The differ ence between immature and mature female rabbits was significant.

Basophil Morphology

Diameters of human basophils ranged between 12.0 and 15.2 microns with an average of 12 6 microns. In the rabbit the measurements were between 10 4 and 15.2 and 13.2 average. Rabbit basophils give with panoptic stains a stronger purple metachromatic colour than human basophils. Young human and rabbit basophils differ in the smear from the peripheral blood in their morphology In the "P" (peripheral) type of human basophils of nucleus usually holds a central position in the cytoplasm and the granules surround it. In the rabbit the cell of corresponding age has its nucleus in an excentric position and the granules are massed at the other side of the cell. In both species, the round or only slightly indented nucleus is clearly visible only few if any granules cover it. The "I" (intermediate) and the U' (uniform) cell types of human and rabbit basophils closely resemble each other (figs. 1 and 2)



Fig Basophil morphology and cell age. Top row human basophils bottom row: rabbit basophils. From left to right "P" I and U" forms.

Cell type and size The diameters of 400 rabbit basophils selected at random were measured in the stained smear and the results compared with the position of the nucleus.

Cell type	P	I	U	All three types
Mean cell	13.9	13.2	12.6	13.2 microns

The difference in cell diameters between peripheral and uniform cell types was significant.

Position of the Granules and Sex

a) Humans In 1451 basophils from 90 male and 100 female healthy human adults positions of the granules were compared with the following results 1 Adult females exceeded adult males in the mean percentage of P" and I" forms. 2 During the menstrual cycle adult females had twice as many basophils of the "P" type shortly before or at the beginning of the menstrual bleeding as in the first post menstrual week. The results are summarized in table II

Table II
Position of the granules of basophils in 190 human adults.

	Cell types per 100 basophils		
	P	I	U
Males	17	18	65
Females	18.5	30	51.5
first week after menstruation	15	32	53
last week after menstruation	26	28	46

The differences were significant in all groups.

b) Rabbits The position of the granules in 2054 basophils were compared between 5 male and 5 female adult rabbits. Adult female rabbits had a significantly higher percentage of basophils of the "P" and "I" type than adult male animals (table III)

Table III
Position of granules of basophils in adult rabbits.

	Cell types per 100 basophils		
	P	I	U
Males	6.6	18.1	75.3
Females	8.5	25.4	66.1

The differences between male and female rabbits were highly significant ($p < 0.01$) for each group.

In one male and one female immature rabbit the positions of the granules were compared in 449 cells. No statistical difference was found in the relative frequency of the "P" type cell between the two animals.

Discussion

Basophil numbers The data submitted suggest that the number of human and rabbit basophils in the peripheral blood are influenced by sex and age. Young female human adults had higher means of basophils than young males of the same age. Among geriatric subjects the ratio was inverted here the female subjects had lower means than their male counterparts. While in the rabbits the groups of animals studied were of a different composition than the human populations, the results were equally suggestive of a sex linked difference in the levels of basophils in this animal. Here it was the sexually mature female which had higher means of basophils than the immature female animal. In the two widely different age groups of human male adults the means of the basophils were identical in spite of different levels of white cell numbers. In the rabbit immature and mature male animals showed no significant differences. In the mature animals it was the female which had the higher means but as BOSELLA (7) had observed, due to the wide variations in the individual counts these differences were not significant. Previous investigators (8) who studied smaller and less homogeneous human populations did not find any sex linked differences with the exception of ANGELI et al. (9) They obtained similar results among 40 geriatric subjects, the females had significantly

lower numbers of basophils. The authors considered the possibility of a relation between basophils and female hormonal activity.

Basophil morphology. For the study of basophils in the stained smear it is essential that fixing and staining be combined in one single process and that in the first stage of staining a completely waterfree technique be used. Otherwise due to their solubility in alcohol and water part of the granule contents will be washed out. Lack of such a water free technique may have been responsible for the differences GRAF AND SWENSSON (10) found between basophil numbers in smears stained with PAPPENHEIM or toluidine blue.

Estimation of cell age by the shape of the nucleus of basophils in the lege artis stained smear is often impossible because the granules are numerous, gross and cover the nucleus. Frequently the lobules of the nucleus are folded over each other giving the impression that it is compact and round. The position of the nucleus its shape and its size in relation to cell volume determine to a large extent the position of the granules. Therefore, in using the different positions of the granules for the estimation of cell age one follows in reality ARNETH's stages of nuclear development. In addition to the nucleus the number of granules may influence their position. In the bone marrow the earliest identifyable stage of basophil development the promyelocyte, has few granules. Their number increases during cell maturation and appears to be greatest in the mature "U" type basophil. But in the peripheral blood other factors besides cell age determine the number of granules. One therefore, may find mature basophils in which a few granules are spread over the whole cell surface. That basophils with their granules at the cell periphery are the youngest of normal circulating basophils and correspond to the metamyelocytes or juveniles of the neutrophils can be deduced from the resemblance to their immediate predecessor the basophilic myelocyte. This latter cell, however when seen in the bone marrow smears has the nucleus frequently in an excentric position while the juvenile or "P" form of human basophils has a central nucleus. In the rabbit, on the other hand basophilic myelocyte and juvenile "P" form have both an excentric nucleus (figs. 1 and 2).

Young leukocytes are usually larger than older cells. The greater diameters of rabbit basophils of the "P" type and the fact that in human and rabbit they constituted the smallest group of circulating basophils point towards their being the youngest normal baso-

phils of the peripheral blood. Shift to the left with an increase in "P" and "I" types of basophils occurred frequently during intercurrent diseases of the rabbits. They preceded or accompanied rises in total basophilic numbers. Only in two occasions, during a fatal illness a shift to younger basophils was seen during a falling total basophil count.

Among 1500 human basophils from normal subjects 15 % were of the "P" type, and 28 % of the "I" type. Among over 9000 rabbit basophils the corresponding proportions were 7 and 22 %. One of the reasons for the permanent presence of a higher proportion of young basophils than of young neutrophils in the blood stream is the small number of basophils. Because of a smaller supply base the need for replacement of basophils results in the release of more immature forms from the bone marrow. For the same reason the shift to younger forms is more pronounced among human basophils than in rabbit blood because man has much less basophils than the rabbit.

Female sexual maturity seems to be one of the factors which influence basophil economy. The higher means of basophils and their more rapid turnover in young mature female humans and mature female rabbits point in this direction. There is further evidence which correlates quantitative and qualitative changes in basophils with different degrees of ovarian activity (11). In addition there are other endocrine systems whose functional state produces alterations in the levels of circulating basophils. The effects of the secretions of thyroid and suprarenal cortex have been studied and are well substantiated (2). The data presented in this study show that the basophil differential count is a more sensitive indicator of basophil movements than their numbers alone. Alterations in basophil morphology may, therefore, be washed with profit for the study of changes in basophil economy under physiologic conditions.

Summary

Numbers and morphology of circulating basophils of 694 human subjects and 45 rabbits were examined. The position of the granules in the basophils was used to determine cell age. Young adult human females had higher mean of basophil numbers than the males in the same age group. Geriatric females had lower mean than their male counterparts. Sexually mature female rabbits had higher average number of basophils than immature females or mature and immature male animals. Basophil differential counts showed that young adult female humans and mature female rabbits had the fastest turnover of basophils among the groups examined.

Résumé

On étudia le nombre et la morphologie des basophiles circulants chez 694 personnes et chez 45 lapins. L'âge des cellules fut déterminé d'après la localisation des granules dans les basophiles. En moyenne on trouva chez les femmes jeunes plus de basophiles que chez les hommes du même âge. Chez les femmes âgées on en trouva moins. Chez les lapines pubères on trouva plus de basophiles que chez les lapines impubères et que chez les lapins pubères et impubères. Le compte différentiel démontra que dans les groupes étudiés le turnover le plus intense se trouve chez les femmes jeunes et chez les lapines pubères.

Zusammenfassung

Bei 694 Versuchspersonen und 45 Kaninchen wurden Zahl und Morphologie der zirkulierenden Basophilen untersucht. Die Lage der Granula in den Basophilen wurde zur Bestimmung des Zellalters herangezogen. Jugendliche Frauen zeigten höhere Durchschnittswerte der Basophilenzahl als Männer derselben Altersklasse. Im Greisenalter wiesen Frauen niedrigere Werte auf als Männer. Geschlechtsreife weibliche Kaninchen hatten eine höhere Basophilenzahl als unreife Weibchen oder reife und unreife Männchen. Differentialzählungen ergaben, daß der Umsatz der Basophilen in den untersuchten Gruppen bei jugendlichen Frauen und reifen weiblichen Kaninchen am größten war.

References

1. Moore, J. E. and James, G. W. A simple direct method for the absolute basophil leukocyte count. Proc. Soc. exp. Biol., N. Y. 82: 601 (1953).
2. Thonnard-Neumann, E. The influence of hormones on the basophilic leukocytes. A review. Acta haemat. 25: 261 (1961).
3. Arneth, F. Über das Mastzellenblutbild. Berl. klin. Wschr. 1920, 109.
4. Jucker, P. Der Leukozytensturz nach Heparininjektion beim Kaninchen und beim Menschen. Seine Bedeutung für die Bluttransfusion. Klin. Wschr. 22: 221 (1943).
5. Thonnard-Neumann, E. Basophile Leukozyten und Steroidhormone. Medizinische 27/28: 1087 (1958).
6. Inagaki, S. The relationship between circulating basophils and thyroid function. Acta endocrin. 26: 477 (1957).
7. Bonilla, A. W. A. Normal count and physiological variability of rabbit blood basophils. Experientia 15: 149 (1959).
8. Kovács, G. S. A simple direct method for the absolute basophil and eosinophil counts from the same blood sample. Folia haemat. N. F. 5: 166 (1961).
9. Angeli, G.; Tedeschi, G. e Cavazzuti, F. Studio sul comportamento dei basofili ematici in soggetti di età avanzata. Acta geront. 5: 28 (1955).
10. Graf, W. and Swenson, A. Experimental investigation on local changes in the white blood cell picture following perforating injury to blood vessels (veins). J. Path. Bact. 62: 419 (1950).
11. Thonnard-Neumann, E. Ovarian activity in the rabbit and basophilic leukocytes (to be published).

Author's address: Dr. E. Thonnard, St. Elizabeths Hospital, Washington 20, D.C. (USA).

Acta haemat. 30: 229–235 (1963)

From the Institute of Clinical Medicine, University of Freiburg, Br.
(Director: Prof. L. Heilmeyer)
Department of Immunopathology: Prof. H. Schubothe

Studies on the Antigens of Human Red Cell Ghosts

Differences in the S Proteins of the Adult and the Newborn

By Nicoletta Vulpis

In the preceding paper (6) it was shown that human adult S protein is a mixture of three antigens that stimulate, in rabbits, the production of precipitating antibodies specific for each antigen. This was determined by the quantitative precipitin test and immunological gel diffusion precipitation technique. It seemed desirable to investigate further the properties of the S protein especially in order to establish similarities or differences compared with the S protein derived from stromata of the normal new-born infant. The present investigation was undertaken in an attempt to clarify this point.

Methods

Preparation of S protein solutions: The new-born S protein (SECB) was prepared from haemolysate obtained by mixing 5 specimens of A positive cord blood and using essentially the same method described by Moskowitz et al. (2). Two samples of normal adult S protein (SEN_1 and SEN_2) were also prepared. The S protein concentration was estimated by microkjeldahl analysis, assuming nitrogen content of 14.7 %. SEN and SECB were then diluted with distilled water to give final product containing 30 mg N.

Preparation of antisera. Antisera to SECB ($anti_1$-SECB and $anti_2$ SECB) and SEN ($anti$-SEN_1) were produced by immunizing young rabbits by published procedure (6). A total dose of 18.50 mg of SECB and SEN was injected into each rabbit. Non specific antibodies to Hb and serum proteins were removed by addition of small amount of haemoglobin and normal serum. Crystalline oxyhaemoglobin was obtained by using the same method described previously (4). The titer of the antisera was determined by the *interfacial technic.* A gamma-globulin fraction which was obtained by bringing specific volume of antiserum to ⅓ saturation with ammonium sulfate as described (3) as used as the source of antibodies in some experiments.

Immunochemical techniques: The procedure followed for the quantitative precipitin test was carried out as previously described (1). Micro-double diffusion plate tests in agar gel were set up on microscope slides using smaller cutters than they are in Petri dishes

This work was carried out while the author was NATO grantee in Germany

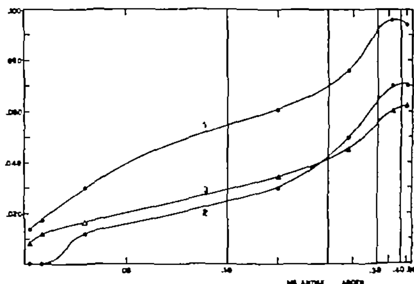


Fig 3. Quantitative immunochemical curves showing cross reaction of SEN with the homologous anti-SEN serum (curve 1) and heterologous anti$_1$-SECB and anti-SECB immune sera (curve 2 and 3 respectively)

with SECB these lines were not continuous, indicating that serologically SEN_1 and SECB are unrelated. On the other hand, the anti$_{1+2}$-SECB and anti-SEN_1 sera gave two continuous lines with SECB and only one continuous line with SEN_1. It can be presumed that both SEN_1 and SECB contain two common *identical* antigens moreover SEN_1 contains a third antigen by which it is characterized. It is also possible that the two common identical antigens are not present in the same amount in SEN and SECB likely one of these occurs in SEN in such small quantities that it may not be detected by the anti$_{1+2}$-SECB immune serum.

Absorption procedures confirmed these observations. Fig 5a shows that the SEN_1-antiserum when exhaustively absorbed with

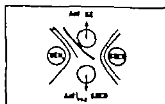


Fig 4 Schematic representation of double-diffusion test showing SEN and SECB to be partially identical but SEN to have an antigenic determinant not present on SECB.

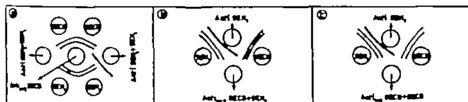


Fig. 5. Comparative double-diffusion analysis after absorption with SEN and SECB. a) When anti-SEN is absorbed with SEN no precipitin band develops neither with SEN nor with SECB; when absorbed with SECB only the uncommon antigenic determinant is shown. b) The precipitin bands developed by anti$_{1+2}$-SECB with SEN and SECB disappear after absorption with SEN. c) The same results are obtained when anti$_{1+2}$S-ECB is absorbed with SECB.

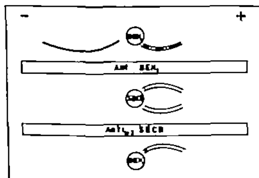


Fig. 6. Comparative immunoelectrophoretic analysis of SEN using anti-SEN$_1$ and anti$_{1+2}$-SECB immune sera. SECB contains two immunoelectrophoretic fractions serologically identical with two of the three SEN$_1$ fractions.

SEN$_1$ was no longer capable of reaction with either SEN or SECB; the same immunserum absorbed with SECB formed with SEN$_1$ one band alone. Besides, the anti$_{1+2}$-SECB serum absorbed with SEN$_1$ or SECB did not give any precipitin reaction neither with SEN$_1$ nor with SECB (fig. 5b, c).

These results are in close agreement with the findings from immunoelectrophoresis which revealed that both SEN$_1$ and SECB contain two equal components in the alpha$_2$-globulin zone, whereas SEN contains a third component situated in the gamma-globulin region (figs. 6, 7a, b, c). No differences were observed when the gamma-globulin preparation was used for comparative study.

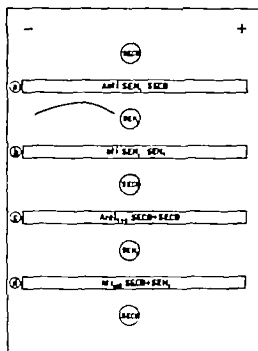


Fig. 7. Comparative immunoelectrophoretic analysis of SEN and SECB using absorbed anti-SEN and anti-SECB immune sera. a) After absorption with SECB anti-SEN produces only with SEN a single precipitin band. b) After absorption with SEN no precipitin band is formed neither with SEN nor with SECB. c) and d) No precipitin reaction is also obtained when anti-SECB is absorbed with SECB or SEN.

Comments

The results reported here suggest that the adult normal S protein differs antigenically from the S protein of the normal new born infant. Using antisera as an analytic agent, the former can be distinguished from the latter by a third antigenic constituent whose line of reaction with homologous antiserum cross clearly the lines given by the new born S protein in agar diffusion tests, thus indicating that serologically they are unrelated.

The question whether this antigenic component is already present at birth but in such low concentration that it escapes detection or it appears only after birth as maturity approaches, has not at present a definite answer. Further work must be done before any conclusion can be made.

Acknowledgments: The author wishes to express her sincere appreciation for the hospitality of Prof. L. HEILMEYER and Prof. H. SCHUBOTHE, whose helpful discussions and critical suggestions have been a great stimulation to this work.

Summary

By means of immunoelectrophoresis and agar-gel double diffusion it was observed that adult S protein differs from the normally occurring S protein of the red cells of new-born infants. At birth only two antigenic determinants are detectable. As maturity approaches a third determinant appears that was shown to have an antigenic specificity.

Résumé

On put démontrer par les méthodes d'immuno-électrophorèse et de double diffusion dans un milieu gélifié (agar) que la protéine S des adultes diffère des protéines S normalement trouvées dans les hématies du nouveau-né. A la naissance on ne peut discerner que 2 déterminants antigéniques. A l'âge mûr un troisième déterminant apparaît de pouvoir antigénique spécifique.

Zusammenfassung

Mit Hilfe der Immunelektrophorese und der Doppeldiffusion im Agar-Gel konnte gezeigt werden, daß das S-Protein des Erwachsenen sich von dem normalerweise vorkommenden S-Protein der Erythrocyten des Neugeborenen unterscheidet. Bei der Geburt lassen sich nur zwei Antigen-Determinanten nachweisen. Bei Erreichen der Reife erscheint eine dritte Determinante die Antigen-Spezifität besitzt.

References

1. GRABAR, P. La technique immuno-chimique quantitative et son emploi en immunologie et en chimie. Techniques de laboratoire (Masson, Paris 1954)
2. MOSKOWITZ, M.; DANDLIKER, W. B.; CALVIN, M. AND EVANS, R. S. Studies on the antigens of human red cells. I. The separation from human erythrocytes of a water soluble fraction containing the Rh, A and B factors. J. Immunol. 65: 383–392 (1950)
3. PÉREZ, J. J. Isolément, purification et cristallisation des protéides. Techniques de laboratoire (Masson, Paris 1954)
4. ROCHE, J.; DERRIEN, Y.; REYNAUD, J.; LAURENT, G. AND ROQUES, M. Sur l'hétérogénéité des hémoglobines. I. Technique d'établissement des courbes de solubilité et premiers essais de fractionnement. Bull. Soc. Chim. biol. 36: 51–63 (1954)
5. SCHEIDEGGER, J. J. Une micro-méthode de l'immuno-électrophorèse. Int. Arch. Allergy appl. Immunol. 7: 103–110 (1955)
6. VULPIS, N. AND VULPIS, G. Etudes sur la constitution antigénique du stroma normal par des méthodes de précipitation spécifique en milieu gélifié et d'immuno-électrophorèse. Constitution antigénique de la protéine N. Rev. franç. Hémat. 2: 333–350 (1962).
7. WADSWORTH, C. A microplate technique employing a gel chamber compared with other micro- and macroplate techniques for immune diffusion. Int. Arch. Allergy 21: 131–137 (1962)

Author's address: Dr. Nicoletta Vulpis, Via Roma 22, Bari (Italy)

Acta haemat. 30 236–243 (1963).

Aus der I. Medizinischen Klinik und Poliklinik der Johannes-Gutenberg-Universität Mainz (Direktor: Prof. Dr. R. Duesberg)

Weitere Untersuchungen zum Serumproteinfilm an der Oberfläche normaler menschlicher Erythrocyten

Von F. Gramlich und H. E. Müller

Wie wir in vorhergehenden Arbeiten (1, 2, 3) zeigen konnten ist die Oberfläche normaler menschlicher Erythrocyten von einem Plasmaproteinfilm überzogen, der sich in seiner Zusammensetzung wesentlich von der Zusammensetzung der Proteine im Plasma unterscheidet. Weiterhin verschieben sich diese Proteinrelationen unter pathologischen Bedingungen für einzelne Krankheitsbilder in charakteristischer Weise (4).

Die Untersuchungen waren an den rein erhältlichen Human-Plasmaproteinen Albumin, *Fibrinogen*, *gamma-Globulin*, *alpha₂-Makroglobulin*, *Präalbumin* und Transferrin durchgeführt worden, außerdem an Lipoprotein, das durch Gelfiltration von Human-Serum leicht in reiner Form darstellbar ist (5).

Bei den so untersuchten Proteinen konnte eine mögliche Denaturierung, bedingt durch ihre Reindarstellung, längere Lagerungsdauer und Markierung mit ^{131}Jod nicht ganz ausgeschlossen werden. Dem hieraus bleibenden Einwand gegen die Ergebnisse dieser Studien suchten wir in der vorliegenden Arbeit folgendermaßen zu begegnen. Das zur Untersuchung gelangende Human-Serum wurde unter den denkbar schonendsten Bedingungen bei niederer Temperatur und in möglichst kurzer Zeit (innerhalb von 24 Stunden) mit ^{131}Jod markiert und elektrophoretisch in einzelne Fraktionen aufgetrennt, mit denen die Untersuchungen am Proteinfilm der Erythrocytenoberfläche durchgeführt wurden. Der Vorteil dieser Untersuchung liegt in der schonenden Proteinaufbereitung und in der Tatsache, daß alle Serumbestandteile zu 100% zur Untersuchung kommen, ihr Nachteil ist in der geringen Auftrennung in einzelne definierte Proteine zu sehen.

Das Untersuchungsschema bestand in Anlehnung an die früher durchgeführten Arbeiten in einer Isotopenverdünnungsanalyse des untersuchten Vollblutes, das mit den ^{131}Jod-markierten Proteinen bis zur Gleichgewichtseinstellung zwischen gelösten und an der Erythrocytenoberfläche adsorbierten Proteinmolekülen inkubiert und danach einer Waschprozedur unterworfen wurde. Im Verlauf der einzelnen Waschvorgänge nimmt die Menge der in der Lösung frei gelösten Proteine schneller ab als die Menge der an die Erythrocytenoberfläche adsorptiv gebundenen Proteine. Es wird für verschiedene Proteine verschieden schnell und bei verschiedener Konzentration

eine sogenannte Gleichgewichtsmenge (P_G) erreicht. Sie ist konventionell so definiert, daß im Überstand und an der Erythrocytenoberfläche gleich große Aktivitäten und damit gleich große Proteinmengen vorhanden sein sollen. Die Gleichgewichtsmenge (P_G) wird in % der Ausgangsmenge der jeweiligen Proteinfraktion ausgedrückt. Diesbezügliche theoretische Ableitungen sind an anderer Stelle ausführlich erörtert (2). Aus dem relativen Vergleich der Gleichgewichtsmengen der einzelnen Serumfraktionen ergibt sich die Zusammensetzung des Proteinfilmes an der Erythrocytenoberfläche (P_E). Die Relation zu den entsprechenden Proteinfraktionen im Serum (P_P) wird durch An- bzw. Abreicherungsfaktoren ($F = P_E/P_P$) ausgedrückt.

Die Untersuchungen an reinen Proteinen zeigten eine starke Anreicherung in der Reihenfolge Präalbumin, Lipoprotein, gamma-Globulin, weniger stark von Fibrinogen und alpha₂-Makroglobulin und schließlich eine Abreicherung von Transferrin und Albumin.

Die vorliegende Arbeit beschäftigt sich mit Proteinfraktionen, die durch Vertikalelektrophorese gewonnen wurden. Nur gamma-Globulin und Albumin konnten dabei in reiner Form hergestellt werden, wie die durchgeführten Immunelektrophoresen zeigen (Abb. 2). Im übrigen liegen Mischungen verschiedener Proteine vor. Deshalb können die Ergebnisse nur an Hand der schon früher gewonnenen Aussagen interpretiert werden. Sie bestätigen die vorhergegangenen Untersuchungen und zeigen, daß die mögliche Denaturierung nur eine sekundäre Rolle spielte.

Material und Methode

Human-Serum wurde nach einer leicht modifizierten Methode von Pressman und Sternberger (5) mit ^{131}Jod (Radiochemical Centre, Amersham) markiert. Die Entfernung des ungebundenen Jods geschah durch Behandeln mit $AgCl_2$ (zweimaliges Rühren des Serums mit gleichen Volumteilen $AgCl_2$ während 30 min) und Durchlauf durch eine Ionenaustauschersäule mit Amberlite IR 45. Nach der $AgCl_2$-Behandlung betrug der freie Jodanteil noch 15 %, nach dem Durchlauf durch die Ionenaustauschersäule weniger als 0,5 % der Gesamtaktivität. Anschließend wurde das jodierte Serum einer Vertikalelektrophorese unterworfen (Vertikalelektrophorese-Apparatur nach Beckman, Modell CP; angelegte Spannung: 600 V; Verdünnung des Serums 1 : 1 mit Dona-Puffer pH 8,6, Durchfluß 4 ml/Stunde). Alle Manipulationen wurden bei 4°C ausgeführt und dauerten insgesamt 20 Stunden. Das Serum wurde dabei in 21 Fraktionen aufgetrennt. In jeder Fraktion wurden der Proteingehalt, die Aktivität und der Anteil an freiem Jod gemessen; er lag auch nach der Durchführung der Vertikalelektrophorese nirgends über 1%.

Zur besseren Charakterisierung der einzelnen Elektrophorese-Fraktionen wurde von jeder einzelnen Fraktion eine Immunoelektrophorese angesetzt nach der Methode von Grabar (6) und Scheidegger (7) bei 110 V, Entwicklungszeit 3 Stunden, Präzipitation durch Antihuman-Serum der Fa. Hyland.

Blut der Blutgruppe 0 eines gesunden, jungen, männlichen Spenders wurde auf ACD abgenommen (Ery 4,18 Millionen/mm³, Hämatokrit 37°, Biuret-Wert 5,6 g%) und in 21 Proben zu je 5 ml aufgeteilt. Die einzelnen Proben wurden mit den 21 Fraktionen des ^{131}Jod-markierten Serums 2 Stunden bei Raumtemperatur inkubiert, wobei zwar gleiche Aktivitäten, aber verschiedene Proteinmengen eingesetzt wurden. Sie wurden folgender Waschprozedur unterworfen. Das mit ^{131}Jod-Protein inkubierte Blut

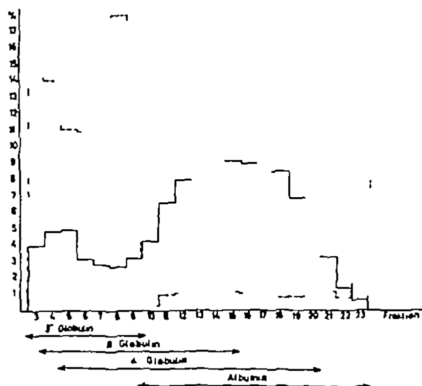


Abb. 1 Die Zusammensetzung der Proteine im Plasma (ausgezogen) und an der Erythrocytenoberfläche (gestrichelt)

wurde mit 0,15 m Sörensen-Phosphat-Puffer pH 7,2 und physiolog. NaCl-Lösung (1:1) auf 10 ml aufgefüllt, 5 min bei 1200 g zentrifugiert und vom Überstand jeweils 5 ml abpipettiert. Nach Zugabe von weiteren 5 ml Phosphatpuffer wurde die Erythrocytensuspension gründlich durchgemischt und erneut zentrifugiert. Die Waschprozedur wurde 15mal durchgeführt und in den gewonnenen Waschüberständen die Aktivität des Jod-Proteins gemessen. Nach beendigter 15. Waschung wurde die Aktivität mit dem restlichen Erythrocytensediment ebenfalls bestimmt.

Ergebnis

Unter Berücksichtigung des sich während der einzelnen Waschvorgänge verändernden Hämatokritwertes (2) läßt sich errechnen, welche Proteinmenge nach jeder einzelnen Waschung noch an der Erythrocytenoberfläche adsorptiv gebunden ist. Die Gleichgewichtsmenge (I a) der Tab. I Spalte 2 ist also dadurch gekennzeichnet, daß im Überstand und im Erythrocytensediment gleiche Proteinmengen vorhanden sind. Im Gegensatz zu vorhergehenden Arbeiten wurden in der vorliegenden Untersuchung die Gleich-

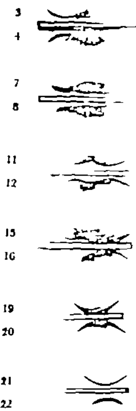


Abb. 2. Immunelektrophoresen der in der präparativen Elektrophorese gewonnenen Serumfraktionen.

gewichtsmengen (P_G) an adsorbiertem Protein nur auf das Erythrocytensediment allein und nicht auch auf die überstehende Lösung bezogen, die bei 5 ml Gesamtvolumen immer noch vorhanden ist. Aus diesem Grund sind die neu erhaltenen P_G Werte nicht völlig mit den Werten der vorhergegangenen Publikationen zu vergleichen.

Setzt man die Gleichgewichtsmengen P_G aller 21 Fraktionen gleich 100 %, so lassen sich Prozentwerte errechnen, die der Serumzusammensetzung an der Erythrocytenoberfläche entsprechen (P_E

der Tab. I Spalte 3) Der Quotient aus P_E und der prozentualen Zusammensetzung des Serums (P_P der Tab. I Spalte 4) gibt den An- oder Abreicherungsfaktor F an, der die Verschiebungen zwischen der Zusammensetzung des Serums und der Zusammensetzung des Serumproteinfilmes an der Erythrocytenoberfläche kennzeichnet.

In Tab. I und Abb. 1 sind die entsprechenden Werte zusammengestellt. In Abb. 1 ist außerdem die Bandenbreite der einzelnen Proteingruppen aus der Vertikalelektrophorese angegeben, wie sie durch immunelektrophoretische Untersuchungen gewonnen wurden (Abb. 2). Hieraus ebenso wie aus Abb. 1 ist zu erkennen, daß praktisch nur die ersten gamma-Globulin- und die letzten Albumin-Fraktionen in der Immunelektrophorese reine Proteine enthielten. Alle übrigen Fraktionen bestehen aus Mischungen von mehr oder weniger vielen Eiweißkörpern.

Tabelle I

Die Proteinzusammensetzung im Serum und an der Erythrocytenoberfläche

1	2	3	4	5
Frak. Nr.	Gleichgewichtsmenge	Proteingehalt am Ery	Proteingehalt im Serum	Anreicherungsfaktor
	P_G	P_E	P_P	$F = P_E/P_P$
3	0,236	17,03	3,936	4,33
4	0,194	14,02	4,786	2,93
5	0,153	11,03	4,893	2,26
6	0,151	10,91	3,191	3,42
7	0,100	7,23	2,766	2,61
8	0,249	17,99	2,6[illegible]9	6,77
9	0,077	5,[illegible]6	3,191	1,74
10	0,004	0,29	4,255	0,07
11	0,013	0,94	6,[illegible]96	0,14
12	0,014	1,01	7,978	0,13
13		—		
14	—			
15	0,016	1,16	9,149	0,13
16	0,015	1,08	9,042	0,12
17	—			
18	0,010	0,72	8,[illegible]11	0,08
19	0,011	0,79	6,914	0,11
20				
21	0,016	1,16	3,297	0,35
22	0,011	0,79	1,382	0,57
3	0,114	8,[illegible]4	0,638	1,[illegible]9

Diskussion

Bei der Interpretation der vorliegenden Ergebnisse muß auf die Resultate der früher veröffentlichten Arbeiten zurückgegriffen werden. Zum besseren Verständnis seien hier die entsprechenden Werte (3) in Tab. II angeführt Aus Tab I und Abb 1 ergibt sich eine deutliche Anreicherung von gamma-Globulin an der Erythrocytenoberfläche sie ist charakteristischerweise in der reinsten gamma-Globulin-Fraktion (Fraktion Nr 3) am größten und sinkt etwa in dem gleichen Maß wie andere Proteine (immunelektrophoretisch Proteine der beta und alpha Globulin Reihen) in den Fraktionen erscheinen Der hohe Peak bei Fraktion 8 ist nur unter

Tabelle II

Die Proteinzusammensetzung des Plasmas und der Erythrocytenoberfläche einiger definierter Proteine.

1	2	3	4
Protein	Proteingehalt am Ery P_E	Proteingehalt im Plasma P	Anreicherungsfaktor $F = P_E/P$
Albumin	7,51	60	0,13
gamma-Globulin	38,72	16	2,42
Fibrinogen	8,42	4	2,11
Lipoprotein	29 17	5	5,83
alpha$_2$-Makro-globulin	5,46	4,5	1,21
Präalbumin	4,57	0,5	9 13
Transferrin	0,79	5	0 16

Zugrundlegung der Ergebnisse aus Tab. II zu verstehen. Vermut lich handelt es sich an dieser Stelle um alpha oder beta-Lipoproteine, u. U auch Mucoproteine, die eine so starke Anreicherung an der Erythrocytenoberfläche erfahren Dafür spricht der hohe Anreicherungsfaktor F für Lipoproteine aus Tab II, doch kann ein strenger Beweis dafür bei der Vielzahl der immunelektrophoretisch in dieser Fraktion nachgewiesenen Proteine nicht erbracht werden Im Übrigen scheinen sowohl in der Gruppe der alpha wie auch der beta-Globuline keine Einzelproteine vorzuliegen die eine extrem starke Anreicherung an der Erythrocytenoberfläche erfahren Das wird durch die Einzelbeobachtungen an alpha$_2$ Makroglobulin und am Transferrin der Tab. II bestätigt. Ebenso wie am Reinalbumin der Tab. II konnte auch jetzt an Elektrophoresefraktionen die Abreicherung von Albumin an der Erythrocytenoberfläche gegenüber der Konzentration im Serum bestätigt

werden. Großes Interesse beansprucht der hohe Anstieg der Proteine aus der Fraktion 23. Ein Vergleich mit den Werten der Tab. II berechtigt zu der Annahme, daß es sich hier um einen Präalbumin-Effekt handelt. Wie Immunelektrophoresen mit reinem Präalbumin zeigten, spricht das verwendete Antihuman-Serum nicht auf Präalbumin an, deshalb erscheint in der entsprechenden Immunelektrophorese keine Präalbumin-Bande. Daß es sich andererseits nicht um einen Albumin-Effekt handeln kann, wird durch die vorhergehenden, immunelektrophoretisch ebenfalls reinen Albumin-Faktoren bestätigt.

Abschließend läßt sich auf Grund der vorliegenden Ergebnisse folgendes feststellen: Die Zusammensetzung des Proteinfilmes an der Erythrocytenoberfläche unterscheidet sich wesentlich von der Zusammensetzung im Serum. Dabei werden gamma-Globulin, Lipoprotein und Präalbumin in besonders starkem Maß an der Erythrocytenoberfläche angereichert, während die Erythrocytenoberfläche an Albumin deutlich verarmt. Die An- und Abreicherungsfaktoren entsprechen größenordnungsmäßig den bereits in früheren Arbeiten gefundenen Werten.

Fräulein E. [illegible] danken wir für wertvolle Mitarbeit. Der Deutschen Forschungsgemeinschaft danken wir für ihre Unterstützung.

Zusammenfassung

Auf der Erythrocytenoberfläche befindet sich ein adsorptiv gebundener Plasma-Protein-Film. Seine prozentuale Zusammensetzung zeigt gegenüber der Zusammensetzung der im Plasma frei gelösten Proteine wesentliche Verschiebungen. Gamma-Globulin, Lipoprotein und Präalbumin sind an der Erythrocytenoberfläche vermehrt vorhanden, Albumin verringert.

Summary

Bound to the surface of erythrocytes by adsorption is a film of plasma proteins. Its composition differs considerably from that of the free plasma proteins: gamma globulin, lipoprotein and prealbumin are present in increased quantities on the surface of the erythrocytes and albumin is reduced.

Résumé

On trouve absorbé à la surface des hématies, un film de protéines plasmatiques. La répartition des protéines de ce film diffère de celle des protéines plasmatiques pures. On y trouve une augmentation des gamma-globulines, des lipoprotéines et des préalbumines et une diminution des albumines.

Literatur

1. Müller, H. E. und Gramlich, F. Untersuchungen am natürlichen Plasmaproteinfilm der Erythrocytenoberfläche. Acta haemat. 29: 135 (1963).
2. Müller, H. E. und Gramlich, F. Die normale Zusammensetzung des Plasmaproteinfilmes an der Oberfläche menschlicher Erythrocyten. Hoppe Seylers Z. physiol. Chem. (im Druck)
3. Müller, H. E. *und* Gramlich, F. *Ergänzende Untersuchungen zum Plasma*proteinfilm an der Oberfläche normaler menschlicher Erythrocyten. Naturwissenschaften 50: 347 (1963)
4. Müller, H. E. und Gramlich, F. Die Zusammensetzung des Plasmaproteinfilmes an der Oberfläche menschlicher Erythrocyten bei verschiedenen Krankheitsbildern. Blut (im Druck)
5. Pressman D. and Sternberger, C.A. The relative rates of iodination of serum components and the effect of iodination on antibody activity J. amer. chem. Soc. 72: 2226 (1950)
6. Grabar, P. et Williams, C.A. Méthode immunoélectrophorétique d'analyse de mélanges de substances antigéniques. Biochim. biophys. Acta *17*: 67 (1955).
7. Scheidegger, J. J. Une micro-méthode de l'immuno-électrophorèse. Int. Arch. Allergy *7*: 103 (1955)

Adresse der Autoren: Dr. F. Gramlich und Dr. H. E. Müller, I. Medizinische Klinik und Poliklinik der Universität, *Mainz* (Deutschland).

Acta haemat. 30: 244–252 (1963)

From the Departments of Obstetrics and Gynecology and the Department of Pediatrics, College of Physicians and Surgeons, Columbia University and the Presbyterian Hospital, New York, N.Y.

Normal Fibrinolytic System in Two Cases of Familial Hypofibrinogenemia*

By LOUISE LANG PHILLIPS** VALIJA SKRODELIS AND JAMES A. WOLFF

Although an absence of spontaneously active fibrinolysin has been frequently noted in congenital fibrinogen deficiencies, little information has been reported on the other factors of the fibrinolytic system. ASTRUP (1) using his heated fibrin plate method has recently demonstrated normal levels of profibrinolysin in the euglobulin precipitate from the serum of a child with congenital afibrinogenemia.

Congenital afibrinogenemia, a rare abnormality of the coagulation mechanism (2–7) usually manifests itself early in life appears to be a hereditary recessive trait with strong familial tendency and is not sex linked, although males seem to be more frequently affected than females. Often there have been consanguineous marriages in the families of such patients.

Reports on congenital hypofibrinogenemia are less frequent although the incidence is probably higher than that of afibrinogenemia (8–13). Many patients with congenital afibrinogenemia have one or both parents or siblings with fibrinogen levels below 200 mg (? 3 4) Clinical manifestations of congenital hypofibrinogenemia are much less severe or may even be absent so that the condition may be recognized only when specifically looked for

This work was supported by Grant number U 1063 of the Health Research Council of the City of New York and H-1512 from the National Heart Institute, National Institutes of Health, Bethesda, Maryland.

Recipient of Investigatorship of the Health Research Council of the City of New York under contract number I-145.

This report presents two cases of familial hypofibrinogenemia, presumably genetically determined, occurring in a father and his son. Particularly studied was the fibrinolytic enzyme system in relation to the hypofibrinogenemia.

Methods

Fibrinogen was determined by the method of RATNOFF AND MENZIE (14), *free profibrinolysin* by hydrolysis of casein by plasma activated by streptokinase (15) *total profibrinolysin* by hydrolysis of casein by an isoelectric precipitate from the same plasma and activated with streptokinase (15)

Inhibitors were calculated from the difference between total and free profibrinolysin (15). These figures represent inhibition of both the activation of the profibrinolysin and hydrolysis by the fibrinolysin resulting from the activation.

Clot lysis was determined by observation of serially diluted plasma clots using modified Coon method (16).

Euglobulin thrombin time was recorded as the clotting time in seconds of the redissolved euglobulin precipitate prepared for total profibrinolysin on the addition of 0.01 ml. thrombin (1000 N. I. H. units per ml)

Other studies included QUICK' prothrombin time (17) proaccelerin (factor V) by the method of STEFANINI (18); prothrombin consumption time by the method of SCHULMAN et al. (19) and the thromboplastin generation test (20)

Electrophoresis of the plasma was carried out using the Spinco Model R paper electrophoresis system.

Case Histories

A. L., 9 ½ year old white boy was referred to the Pediatric Hematology Clinic of this hospital in August 1960 for diagnosis, because routine laboratory studies in another hospital prior to scheduled tonsillectomy had revealed deficien fibrinogen level. Bleeding time, coagulation time, and liver function tests were said to be normal.

The patient had developed normally in spite of frequent upper respiratory infections since the age of one year and an allergy to dust and feathers. No overt bleeding manifestations had been observed. Circumcision and first tonsillectomy at the age of 3 years had been performed without complications or undue bleeding.

Physical examination showed normally developed boy with no systemic abnormalities ex for slight lymphoid enlargement of the posterior pharyngeal wall and tonsillar arches. No signs of an existing coagulation disorder such as petechiae, ecchymosis or excessiv bruising were noted. A repeat tonsillectomy had been advised because of recurrent upper respiratory infections.

Laboratory findings on August 4 1960 showed hemoglobin 11 g/100 ml, WBC 6,00 th normal differential count, platelets 200,000, ESR 3 mm/hr Bleeding time 40 seconds. LEE WHITE venous clotting time 15 minutes, tourniquet test negative, fibrinogen 140 mg%. Urinanalysis negative. Clot retraction was poor

The family history showed that the only member of the family with suggestion of hemorrhagic difficulties was the patient father J L. He reportedly had had several bleeding episodes during his life, after circumcision, after tonsillectomy at the age of 8, and after an operation on the nasal septum in 1944 H had also suffered stroke in 1956. His enous clotting time was found to be 13 minutes.

The patient mother L. L., as well as his two brothers, S. L., aged 15 years, and P. L., aged 3 years, were all living and well and denied any bleeding tendency The patient grandparents on both sides of the family had not considered themselves "bleeders There as no consanguinity

A tentative diagnosis of familial hypofibrinogenemia in the patient was made. Subsequent studies confirmed the initial diagnosis.

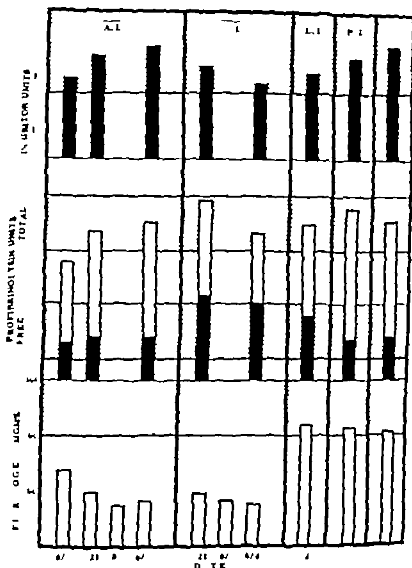


Fig. 1. Fibrinogen, profibrinolysin and inhibitor levels found in the plasma of members of the family. Cross-hatched areas represent normal ranges for the various factors.

Results

Fibrinogen levels were determined on all members of the family for the first time on September 21, 1960. The mother and both brothers of the patient had fibrinogen levels within the low normal

range, but both the patient and his father had definitely decreased levels, 98 mg / and 94 mg / respectively Variations were minimal, indicating that the condition was persistent. The results are shown in figure I

Levels of free and total profibrinolysin as well as inhibitors are also shown in this figure. These determinations were made three times on the patient, twice on his father and once on the other members of the family It should be noted that these results were within essentially normal ranges (cross hatched area) on all members of the family

Tests for spontaneous fibrinolytic activity as measured by the modified Cook's test and for spontaneous caseinolytic activity were negative at all times in all members of the family

Euglobulin thrombin times were consistently prolonged in the patient and his father ranging from 22 to 43 seconds, but normal in the patient s mother and brothers (10–13 seconds) Prolongation of the thrombin time may be indicative either of anti-thrombin activity or may be due to the low fibrinogen concentration in the euglobulin precipitate.

A comparison of the results of various tests of the fibrinolytic system in familial hypofibrinogenemia in these two cases and in acquired hypofibrinogenemia, as found in severe abruptio placentae, metastatic carcinoma, and surgical hemorrhage, are shown in table I. The most significant differences lie in the decreased levels of total profibrinolysin and inhibitors. Active clot lysis or caseinolysis may sometimes be demonstrated in acquired cases.

Other coagulation studies were performed on the patient and his father in October 1960 and again in June 1961 (table II) Prothrombin times were slightly prolonged on both occasions, possibly due to the low plasma fibrinogen levels. Factor V (proaccelerin)

Table I
Hypofibrinogenemia.

	Hypofibrinogenemia	
	Acquired	Familial
Fibrinogen	Low	Low
Free profibrinolysin	Normal	Normal
Total profibrinolysin	Low	Normal
Inhibitors	Low	Normal
Clot lysis	Often demonstrable	No lysis
Euglobulin thrombin time	Prolonged	Prolonged
Euglobulin lysis time	Often rapid	Normal
Spontaneous caseinolysis	Often present	Negative

Table II

		[illegible]		[illegible]	
		A. L.	J. L.	A. L.	J. L.
Prothrombin time seconds, (control: 14)		17	16	17	16
Factor V		70	75	73	80
Prothrombin consumption time, seconds		17	17	20	21
Thromboplastin generation	Incubation time minutes	Coagulation time seconds			
	1	38	34	44	26
Patient's plasma	3	17	18	15	14
Patient serum	5	18	19	15	14
	7	19	20		
	1	16	16		
Normal plasma	3	16	17		
Patient's serum	5	18	18		
	7	18	19		
	1	21	21		
Patient's plasma	3	18	20		
Normal serum	5	21	21		
	7	27	29		
	1	22		34	
Normal plasma	3	14		13	
Normal serum	5	15		14	
	7	17		16	

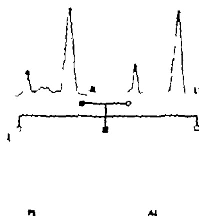


Fig. [illegible] Plasma electrophoretic patterns of members of the L. family. Note the low [illegible] peaks in the patterns of the affected members—father (J. L.) and son (A. L.). Other members show normal plasma electrophoretic patterns.

determinations were normal. Prothrombin consumption times were somewhat short on both occasions. Thromboplastin generation was equivocal in both father and patient in October but normal in June. The original tests suggested a slight anti hemophilic factor defi-

ciency of temporary nature. This was probably reflected also in the impaired prothrombin consumption. These findings agree with those of VAN CREVELD who reported a normal content, but increased lability of anti-hemophilic factor in patients with congenital afibrinogenemia (7)

Electrophoresis of plasma samples (fig 2) gave low fibrinogen peaks in the patient and his father as opposed to the much higher peaks found in the plasma of the mother and both brothers.

Discussion

The coagulation mechanism in patients with congenital hypofibrinogenemia is usually only moderately impaired. Laboratory studies show results similar to those found in congenital afibrinogenemia, but indicate a much less severe depletion of the various clotting factors. Bleeding time may be normal or slightly prolonged, coagulation time is only moderately prolonged as is QUICK's prothrombin time. Other factors—V VII and prothrombin content—are normal thromboplastin generation is normal platelet counts vary and tests for capillary fragility are normal or only slightly positive (9–13) Fibrin clots that are formed are of poor quality and IMPERATO et al. (11) believe that the defect may be qualitative as well as quantitative. As a rule, these patients have little tendency to spontaneous hemorrhages, such as easy bruising and epistaxis so that clinical manifestations of the lowered fibrinogen level may be absent.

Studies of the fibrinolytic mechanism provide an excellent opportunity for comparison with patients who have an acquired hypofibrinogenemia of a temporary nature, as found in association with various disease states—abruptio placentae, dead fetus syndrome' surgery or metastic carcinoma of the prostate. In these cases low fibrinogen levels are usually accompanied by correspond ing decreases in profibrinolysin and inhibitors. This has been interpreted as indicative of increased activation and utilization of the enzyme precursor The resulting fibrinolysin destroys fibrinogen as well or any fibrin clots which may be formed. Active fibrinolysin itself may or may not be demonstrable in vitro. A return to normal levels of all factors of the enzyme system is observed after the hemorrhagic state is controlled and the underlying cause is cor rected (21–23)

In contrast to this picture were the completely normal levels of profibrinolysin and inhibitors in the two patients with familial hypofibrinogenemia despite severely decreased fibrinogen levels. Moreover the variations in the enzyme levels were within normal limits over a period of ten months indicating a stable condition.

Earlier investigators have suggested that the coagulation defect in congenital afibrinogenemia is due to inability of the liver to synthesis fibrinogen in sufficient amounts (5 8) More recently GITLIN et al. (24 25) showed that the turnover of I^{131}-labelled fibrinogen in patients with congenital afibrinogenemia is essentially the same as that found in normal individuals, thus ruling out accelerated removal of fibrinogen from the vascular system. A more rapid turnover has been noted only in a few patients who had developed a precipitating anti-fibrinogen antibody as a result of repeated fibrinogen therapy (26, 27)

Although fibrinogen turnover was not determined in either of the patients presented here, abnormal degradation of fibrinogen did not seem to be taking place. Both were healthy during the study neither father nor son had ever received transfusions or fibrinogen, and in both of them the fibrinolytic system was normal and showed remarkable stability throughout the study period.

The results obtained by ASTRUP (1) and those presented in this report indicate that patients with congenital fibrinogen deficiencies apparently possess completely normal fibrinolytic systems. Therefore it appears unlikely that the low level of fibrinogen has caused a decrease in the production of profibrinolysin or that activation of profibrinolysin is responsible for decreased fibrinogen. Also the levels of the two substances are probably not controlled by the same regulating factor or factors.

These patients would presumably respond in the same way as normal individuals do under conditions which might activate the fibrinolytic system. Combined with their already low fibrinogen reserves this activation might result in serious hemorrhagic difficulties. Therefore fibrinogen should always be available for these patients when surgery or delivery is anticipated.

Summary

Two cases of familial hypofibrinogenemia occurring in a father and his son have been presented. Fibrinogen values ranged from 75–140 mg%. Other members of the family had fibrinogen levels between 200–230 mg%. Studies of the fibrinolytic enzyme

system in father and son showed normal levels of profibrinolysin and inhibitors over a period of 10 months. These results differ markedly from those obtained in cases of acquired hypofibrinogenemia where low fibrinogen is accompanied by correspondingly low levels of profibrinolysin and inhibitors.

Résumé

Deux cas d'hypofibrinogénémie familiale sont décrits chez un père et son fils. Les taux de fibrinogène variaient de 73–140 mg%. Chez d'autres membres de la famille ces taux comprenaient 200–230 mg%. En étudiant le système enzymatique fibrinolytique chez le père et le fils on trouva des taux de profibrinolysine et d'inhibiteurs normaux. Les contrôles furent effectués pendant une période de dix mois. Ces résultats diffèrent nettement de ceux trouvés dans des cas d'hypofibrinogénémie acquise, où l'on constate un taux de fibrinogène diminué et de très basses valeurs de profibrinolysine et d'inhibiteurs.

Zusammenfassung

Zwei Fälle von familiärer Hypofibrinogenämie bei Vater und Sohn werden beschrieben. Die Fibrinogenwerte betrugen 75–140 mg%. Bei anderen Familienmitgliedern lagen sie zwischen 200 und 230 mg%. Die Untersuchungen des fibrinolytischen Enzymsystems bei Vater und Sohn ergaben normale Werte von Profibrinolysin und Inhibitoren über eine Zeitspanne von 10 Monaten. Diese Ergebnisse unterscheiden sich deutlich von den Beobachtungen bei Fällen von erworbener Hypofibrinogenämie, wo ein erniedrigtes Fibrinogen zusammen mit entsprechend niedrigen Werten von Profibrinolysin und Inhibitoren vorkommt.

References

1. Astrup T and van Creveld, S. Normal fibrinolytic system in blood in congenital afibrinogenemia and its significance. Thromb. Diath. haem. *6*: 188 (1961).
2. Lawson, H. A. Congenital afibrinogenemia. New Engl. J Med. *248*: 552 (1953)
3. Frick, P G. and Mc Quarrie, I. Congenital afibrinogenemia. Paediatrics *13*: 44 (1954).
4. Hirt, V and Prins, A. Congenital afibrinogenemia. Ann. paediat., Basel *186*: 182 (1956).
5. Vandenbroucke, J. Verstraete, M. et Verwilghen, R. L'afibrinogénémie congénitale. Acta haemat. *12*: 87 (1954)
6. Prichard, R. W and Vann, R. L. Congenital afibrinogenemia. Amer J. Dis. Child. *88*: 703 (1954).
7. van Creveld, S. and Lenz, K. H. Congenitale afibrinogenemie. Maandschr Kindergeneesk. *26*: 227 (1958).
8. Rhaz, E.: Die Fibrinopenie. Z. klin. Med. *130*: 605 (1935).
9. Alexander, E. C. and Baar, H. S. Fibrinogen deficiency as factor in haemorrhagic disease. Arch. Dis. Child. *18*: 146 (1943)
10. Kuhlmann, N. Congenital deficiency of fibrinogen in identical twins. N. Y J. Med. *53*: 1994 (1953).
11. Imperato, C. Dettori, A. G. Ipofibrinogenemia congenita con fibrinoastenia. Helv Paediat. Acta *13*: 380 (1958).
12. D'Amiano, G. III and Searcy R. L. Congenital hypofibrinogenemia. Laboratory evaluation of case. Amer Osteopath. Ass. *58*: 492 (1959).

13. Hamers, D. A.; Hoekstra, P. G. and Dekker, M. A. E.: Een geval van congenitale hypofibrinogenemie. Ned. T. Geneesk. 104: 510 (1960).
14. Ratnoff, O. D. and Menzie, C.: A new method for the determination of fibrinogen in small samples of plasma. J. Lab. clin. Med. 37: 316 (1951).
15. Phillips, L. L. and Skrodelis, V.: The fibrinolytic enzyme system in normal, hemorrhagic and disease states. J. clin. Invest. 37: 965 (1958).
16. Cook, W. W. and Hodgkiss, P. E.: Fibrinolysis in surgery patients. Surg. Gynec. Obstet. 95: 717 (1952).
17. Quick, A. J.: The Physiology and Pathology of Hemostasis. Philadelphia (Lea & Febiger, Philadelphia 1951).
18. Stefanini, M.: New one-stage procedures for the quantitative determination of prothrombin and labile factor. Amer. J. clin. Path. 20: 233 (1950).
19. Sherman, L. N.; Cohen, I. B. and Gottlieb, R.: Clinical application of simplified serum prothrombin consumption test. J. amer. med. Ass. 156: 702 (1954).
20. Duckert, F.; Flückiger, P.; Isenschmid, H.; Matter, M.; Vogel-Meng, J. and Koller, F.: A modification of the thromboplastin generation test. Acta haemat. 12: 197 (1954).
21. Phillips, L. L.: Etiology of afibrinogenemia: Fibrinogenolytic and fibrinolytic phenomena. Ann. N. Y. Acad. Sci. 75: 676 (1959).
22. Phillips, L. L.; Skrodelis, V. and Furey, C. A.: The fibrinolytic enzyme system in prostatic cancer. Cancer 12: 721 (1959).
23. Tagnon, J. S. and Phillips, L. L.: Fibrinolytic hemorrhage in surgical patients. Surg. Gynec. Obstet. 114: 333 (1962).
24. Gitlin, D. and Borges, W. H.: Studies on the metabolism of fibrinogen in two patients with congenital afibrinogenemia. Blood 8: 679 (1953).
25. Rausen, A. R.; Cruchaud, A.; McMillan, C. W. and Gitlin, D.: A study of fibrinogen turnover in classical hemophilia and congenital afibrinogenemia. Blood 18: 710 (1961).
26. Bachmann, R.: Kongenitale Afibrinogenämie. Acta haemat. 11: 40 (1954).
27. De Vries, A.; Rosenberg, T.; Kochwa, S. and Boss, J. H.: Precipitating antifibrinogen antibody appearing after fibrinogen infusions in a patient with congenital afibrinogenemia. Amer. J. Med. 30: 486 (1961).

Authors' address: Drs. L. Lane Phillips and V. Skrodelis, Dept. of Obstetrics and Gynecology, Presbyterian Hospital, 622 West 168th Street, New York 32, N.Y.; Dr. J. A. Wolff, Dept. of Pediatrics, Babies Hospital, New York, N.Y. (USA).

Progress in Allergy Vol. 7 Edited by *P. Kallós* and *B. H. Waksman*. S. Karger AG, Basel/New York 1963. XII + 334 S., 46 Abb., 42 Tab., Preis sFr. / DM 60.—.

In einer kurzen Einleitung weist B. H. Waksman auf das außerordentliche Wachstum der Basisforschung und der angewandten Forschung auf dem Gebiete der Immunologie hin und gibt einige Hinweise auf besonders aktuelle Forschungsrichtungen.

Über die vererbten Gamma-Globulin-Gruppen beim Menschen, deren Interpretation und Anwendung, berichtet H. Fudenberg. Er weist auf die mögliche große klinische und legal-medizinische Bedeutung dieser erst seit einigen Jahren bekannten Befunde hin.

Einen ganz andern Aspekt der Rolle der Gammaglobuline zeigt K. Ishizaka. In «Gamma Globulin and Molecular Mechanism in Hypersensitivity Reactions» werden die neuen Forschungsergebnisse in extenso besprochen, insbesondere die Bedeutung der löslichen Antigen/Antikörper Komplexe für das allergische Geschehen.

Probleme der Organspezifität mit besonderer Berücksichtigung der Linse werden von S. P. Halbert und W. Manski dargelegt. Die großen Untersuchungsreihen der Autoren über das Verhältnis zwischen Art- und Organspezifität werden ausführlich diskutiert. Ebenso werden Fragen der ontogenetischen Entwicklung der Linse und deren Hemmung durch homologe Antiseren besprochen. Nach einem Hinweis auf die möglichen Zusammenhänge mit klinischen Erscheinungen werden summarisch weitere Probleme über Organspezifitäten, mit besonderer Berücksichtigung der Phylogenese, aufgeworfen.

Die Regulation der autonomen Mikrozirkulation wird von R. W. Schayer aufgezeichnet. Die Hauptrolle wird dem «induced Histamine» zugeordnet, als ausgleichender Antagonist werden die Glucocorticoide betrachtet. Die übrigen möglichen Regulationssubstanzen werden auf Grund der neuesten Forschungsresultate ausgeschlossen.

Das große Kapitel über immunologische Aspekte der Mykosen ist nicht nur für den Immunologen, sondern auch für den Kliniker sehr aufschlußreich. S. B. Salvin führt darin unter anderem die vielen möglichen immunologischen Nachweismethoden auf.

Sämtliche Kapitel sind mit großen Bibliographien versehen. Sowohl für den Wissenschafter wie auch für den Kliniker ist dieser neue Band der Serie «Progress in Allergy» ein wertvolles Arbeitsinstrument und gibt einen ausgezeichneten Einblick in aktuellste Allergie-Probleme.

F. Ott, Zürich

Alexander S. Wiener and Irving B. Wexler: **An Rh-Hr Syllabus.** Second Edition. Grune and Stratton, New York 1963. 108 p., 25 tab., 7 fig. Price $ 4.50.

The second edition of An Rh-Hr Syllabus closely resembles the first in format and content, differing essentially in the addition of co-author (Irving B. Wexler) and 28 more pages. This glossary of term,s tests and theories is dictionary of the assumptions and definitions employed by the senior author The book reveals no effort to relate Rh serology to advances in biochemistry and immunology Instead the authors omit such data (viz. table 5, p. 15).

An Rh-Hr Syllabus ma "include all aspects of serology and genetics" as claimed the preface, but some aspects are treated differently on separate pages. For example, table 11 (p. 40) implies that the Rh phenotype represents a discrete and known number of genotypes. In contrast, table 13 (p. 46), which unfortunately contains some errors, is a denial of such a possibility

Dr. Wiener obviously knows the Rh story The question uppermost in the mind of this reviewer is whether his assumptions, definitions, and bizarre terminology serve an important function in teaching. To Dr. Wiener, Rho, *Rho*, *Rho*, Rho, R and *r* may be necessary but many students cannot clearly differentiate the six complex distinctions. Dr. Wiener knows and states (p. 14) that univalent antibody should not be construed literally - unfortunately the nature of the term sharply narrows the viewpoint of the student. In justice, of course, one must admit that D, D^u *D*, D^u affords only 33% improvement and that the term "incomplete" antibody is just as misleading as "univalent" The Rh story has indeed suffered seriously from misleading communication. As Swanson (J. amer. med. Ass. *185*: 21, 1963) suggests, this dismal fate may await all areas in which data accumulate at a rate in excess of an unbiased coding capacity Blood group serology twenty years ago was in less difficulty; that was 1943, when the third edition of Wiener "Blood Groups and Transfusion" was published. Now reprinted, this book affords a refreshing reminder of Wiener's earlier abilities to communicate.

Richard E. Rosenfield, New York, N. Y.

Acta haemat. 30: 255–270 (1963)

Section of Medicine and Section of Clinical Pathology Mayo Clinic and Mayo Foundation, Rochester Minn.

Acquired Pure Red Cell Agenesis

Report of 16 Cases and Review of the Literature

By Jakob R. Schmid* Joseph M. Kiely Gertrude L. Pease and Malcolm M. Hargraves

In 1922 Kaznelson (23) described the first case of pure red cell agenesis (PRCA) using the syndrome to demonstrate that platelets originated from megakaryocytes as proposed by the theory of Wright and that platelets were not the end result of the expulsed normoblastic nucleus, as suggested by Schilling. In Kaznelson's (23) case, platelets were present in normal numbers and megakaryocytes were plentiful in the bone marrow but normoblasts were absent.

PRCA is a disease of unknown origin, characterized by virtual absence of erythropoiesis in the bone marrow, while granulocytopoiesis and formation of platelets remain relatively unaffected. The disease occurs in two forms, each of which affects a different age group. The congenital form (type of Diamond-Blackfan 11) usually results in death within the first few years of life rarely spontaneous recovery has developed. The chronic acquired form of the disease affects mainly individuals more than 50 years of age. In the present study we will attempt to separate reported cases of the chronic acquired type, of which 23 have been found (11 13, 15, 16, 19 22, 23 28, 29 31–35 37 41 42, 44 48, 49 51) from the congenital variety of which approximately 50 have been reported (1–7 10, 12 14 21 25 27 30 36, 38–40, 47 52, 54)

A separate syndrome has been recognized recently characterized by the association of PRCA with kwashiorkor in marantic children of Kenya (16, 17 26, 53), with almost invariable recovery from the aplasia after riboflavin therapy

In addition to these chronic forms of PRCA, syndrome exists that is characterized by the acute temporary disappearance of normoblasts from the bone marrow This

* Fellow in Medicine, Mayo Foundation.

transient red cell aplasia was described originally by GASSER (18) in association with various toxic and allergic reactions. In this acute type of erythroblastopenia, giant pronormoblasts are noted in the bone marrow. The normoblastic aplasia usually does not last longer than 7 to 10 days.

The chronic acquired disease, which is the subject of the present study appears in two forms the first associated with benign thymoma and the other without this association. We will describe the natural history of the chronic acquired disease, comparing the findings in our own series of patients with findings in previously reported cases. The cases associated with thymoma will be the subject of a separate report. The following terms have been used in the past as synonyms for acquired PRCA pure red cell aplasia or anemia, *primary red cell aplasia*, *erythroblastophthisis*, *idiopathic* aplastic anemia, selective erythroid aplasia, erythroblastic hypoplasia, chronic hypoplastic anemia, essential erythroblastopenia, aregenerative anemia associated with thymoma, and primary refractory anemia.

Material and Methods of Study

Sixteen cases of PRCA were selected from review of the Mayo Clinic files during 10½-year period beginning January 1951 and ending July 1961 For selecting our own cases and those reported in the literature, the following criteria for the diagnosis of PRCA have been used (1) severe normochromic, normocytic anemia, requiring blood transfusions; (2) reticulocyte counts reduced to less than 0.5%, usually 0%; (3) a normocellular bone marrow with selective, virtual absence of the erythrocytic line in the presence of essentially unaltered granulopoesis and adequate numbers of megakaryocytes, and (4) absence of enlargement of the spleen, liver and lymph nodes prior to the onset of hemosiderosis induced by multiple blood transfusions.

Pertinent findings in our 16 cases are summarized in table I and bone marrow differential counts of these cases are listed in table II.

In the literature, 23 cases of acquired PRCA have been reported up to the present time. This series of reported cases is summarized in table III Cases of our series shall be referred to by Arabic numbers and the cases from the literature shall be referred to by Roman numerals.

Clinical Features and Hematological Findings

The combined series (table I and III) includes 39 patients 25 males and 14 females. The age distribution of PRCA is characterized by three peaks (fig 1) The congenital form of DIAMOND and BLACKFAN occurs in early infancy with approximately 50 cases described to this date (1–7 10 12 14 21 25 27 30, 36, 38–40, 47 50 52, 54) The chronic acquired form of PRCA has a small peak of incidence at the time of puberty while most of the cases are seen after the age of 50 years. In contrast, patients suffering from

Table I
Summary of data on 16 cases of acquired pure red cell agenesis encountered at the Mayo Clinic.

Case	Age (years) and sex	Duration (years)	Comment
1	63 F	2½	Toxic exposure only to tolbutamide; diabetes mellitus cryofibrinogen found 9 months after onset hemolytic anemia prior to death
2	76 M	3	Transfusion reaction cause of death; hemosiderosis
3	15 M	–	Had red cell aplasia during infancy with spontaneous recovery relapse at age 15; splenectomy without benefit requires transfusion every 3 weeks
4	49 M	1	Toxic exposure to benzene; concurrent toxic hepatitis cause of death
5	73 F	3½	Accidental death after 125 blood transfusions
6	56 M	–	Toxic exposure to volatile solvent; no help from adrenal steroids still alive 5 years after onset with signs of hemosiderosis requires transfusions
7	67 F	5	Spontaneous remission after 5 years
8	55 M	3	No help from splenectomy; died of hemochromatosis after 130 transfusions
9	60 M	–	Toxic exposure to insecticides no help from splenectomy
10	34 F	½	Toxic exposure to gamma isomer of hexachlorocyclohexane, recovered after removal of toxic agent within 5 months
11	70 F	1	Toxic exposure to gamma isomer of hexachlorocyclohexane only transient benefit from testosterone death later
12	70 M	–	Considerable benefit from testosterone in maintaining hemoglobin at 8 to 9 grams per cent for one year while on androgen therapy
13	72 M	¾	Spontaneous recovery after 9 months
14	56 M	2	Died of acute leukemia hemosiderosis
15	47 M	1½	Toxic exposure to pentachlorophenol; died of acute leukemia
16	55 M	–	Recovered after 3 years from PRCA (112 transfusions) then transition to chronic granulocytic leukemia, still alive

classic aplastic anemia, with depression of all bone marrow cells, are predominantly young adults. In the series of SCOTT et al. (43) 30 of the 39 patients were less than 40 years of age.

Patients with uncomplicated PRCA in our series presented normochromic and normocytic red cells in the peripheral blood smear with no evidence of regeneration. The differential counts were usually unremarkable (table IV) although mild eosinophilia and/or lymphocytosis were occasionally present. Immature leukocytes were practically never found.

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
1	21 10 50	3	40:1	1.2	0.2	18.8	24.8	21.0	4.8	0.4	11.0	1.4	0.8	0	0	0	0	2.4
2	21 3. 57		40:1	4.0	7.8	20.0	21.0	21.0	0.0	0.2	10.2	1.4	0.4	0	0.2	0.4	0.0	1.2
	3 4 57		125:1	3.0	8.4	17.0	25.0	20.4	4.0	0.4	10.0	1.0	1.0	0	0	0	0	0.0
	2 5. 57	10	100:0	2.0	0.0	10.0	21.0	25.0	5.2	0.2	12.2	2.0	2.0	0	0	0	0	0
	20. 8. 57	11	500:0	2.4	8.8	15.0	21.2	21.4	15.4	0.4	15.0	4.0	0.2	0	0	0	0	0.2
	7 5 50	2	45:1	2.0	10.2	14.2	10.0	14.8	2.0	1.2	40.0	2.0	0.2	0	0	0	0	1.0
3	18. 12 50	4	71:1	1.8	2.0	0.2	19.2	35.0	7.0	0.2	17.2	7.0	0.4	0	0	1.0	0.4	0
4	20. 11. 50	4	165:1	3.0	8.0	15.0	12.4	20.2	6.0	0.6	22.4	5.0	0.2	0	0	0	0	0.0
5	2. 10. 56	2	55:1	1.0	2.2	7.2	15.0	10.0	17.4	2.2	30.4	3.0	0.4	0	0.2	0.4	0.8	0.4
6	23. 5. 50	3	18:1	0.0	0.8	3.0	10.0	31.4	11.4	0.0	27.0	1.0	0	0	0.2	0.0	2.8	1.4
7	12. 1 51	3	45:1	0.2	1.0	0.0	10.4	57.2	4.4	0.2	23.0	3.4	0.2	0	0	0.0	1.4	0.2
8	28. 9 51	4	57:1	1.0	3.0	20.4	17.4	23.2	3.0	1.4	24.4	2.0	0.4	2.0	0.6	0	0	0
	17 7 52	4	45:1	0.4	2.0	0.0	15.0	20.0	9.0	0.0	30.0	1.2	2.4	1.4	0.0	0	0	0
9	16. 7 59	2	40:1	1.2	1.8	3.0	14.0	17.2	2.8	0.4	02.4	4.0	1.0	0	0.8	0.0	1.0	0
	15. 2 60	1	50:1	0.0	1.0	1.0	7.8	20.2	0.2	0.2	47.2	10.0	0	0	0.2	0.2	0.8	0.0
10	20. 11 57		90:1	1.0	2.4	13.0	17.0	32.0	0.0	0.4	21.0	1.4	0.0	0	0	0.4	0.0	0
11	10. 2. 61	12	18:1	1.0	4.2	21.2	32.2	10.2	5.4	0	0.2	4.0	0	0	0	1.4	3.2	0.0
	13. 3. 61	17	7:1	1.0	4.0	15.4	27.4	27.0	1.8	0	7.4	2.2	0	0	0.2	2.0	7.6	3.2
12	21 4 60	7	2.0:1	1.0	2.2	11.4	20.4	31.2	5.2	0.2	17.2	1.0	0	0	0.2	0	0.2	0
	8. 7 60	3	100:0	0.8	1.8	14.0	20.4	31.8	5.8	0.0	9.4	2.2	0.4	0	0	0	0	0
	1 11 60		11:1	0	0.0	14.2	20.4	20.2	8.4	0	4.0	0.0	0.2	0	1.2	1.0	0.2	3.0
13	8. 9 61	3	121:1	0.8	3.0	12.4	0.0	20.4	13.0	0.8	30.0	0.0	0.2	0	0.0	0	0	0
14	15 5 61	1	100:0	1.0	0	0	1.0	53.6	2.0	2.0	30.0	2.4	0	0	0	0	0	0
15	11 10. 56	3	55:1	0.0	7.2	20.0	21.0	19.2	0.2	2.2	10.0	0.4	0.2	0.2	1.0	0.2	0.4	0.0
	14 1 57	4	20:1	0.0	4.2	21.0	14.4	12.0	3.0	1.0	17.0	0.0	0.2	0.2	0.4	1.8	2.0	10.0
	8. 8 57	3	25:1	2.2	7.0	14.4	17.4	14.0	2.0	0.2	13.8	2.2	0.8	0.2	0.0	0.8	2.4	23.2
16	4. 12 54	17	124:0	0.8	3.8	13.2	31.6	37.0	0.0	1.4	3.4	0.4	0	0	0.0	0	0	0.2

Table III

Summary of data on 23 cases of acquired pure red cell agenesis reported in the literature.

Case	References	Year	Age (years) and sex	Duration	Comments
I	23	1922	58 M	?	Erythropoiesis absent in marrow at necropsy
II	31	1931	38 M	½ year	Terminal leukopenia
III	22	1937	30 M	6 years	In past, agranulocytosis cancer of breast and hemosiderosis at necropsy
IV	22	1937	21 F	3 years	Amenorrhea since age 19; splenectomy terminal thrombocytopenia
V	33	1942	46 M	3½ years	Hemosiderosis. Death
VI	32	1943	22 F	6 weeks	Died of transfusion reaction
VII	34	1943	20 F	3 years	Temporary leukopenia; was fatal
VIII	49	1943	55 F	6 weeks	Toxic exposure to sulfathiazole recovered after discontinuing drug
IX	37	1950	23 F	2 years	Anemia noted at age 1 improved after puberty relapsed at age 21
X	19	1951	? M	2 years	Toxic exposure to benzol temporary thrombocytopenia
XI	16	1953	46 M	few weeks	Toxic exposure to quinacrine recovered, treated with riboflavin
XII	29	1953	17 M	5 years	Hemolytic anemia developed, recovery after splenectomy and treatment with ACTH
XIII	29	1953	17 M	2 years	Recovery after splenectomy
XIV	44	1953	62 M	1¼ years	Toxic exposure to sulfonamide; agranulocytosis in past. Death from hemochromatosis
XV	44	1953	40 F	5 years	Toxic exposure to dry cleaning fluid recovery with cobalt
XVI	13	1954	58 F	1 year	Auto-immune hemolytic disease developed recovery after splenectomy
XVII	41	1954	51 M	4½ years	Developing hemosiderosis?
XVIII	15	1955	49 F	1½ years	Splenectomy with temporary improvement recovery with cobalt
XIX	51	1957	64 M	3 years	Splenectomy two remissions after treatment with ACTH terminal hemolytic anemia and hemosiderosis
XX	48	1960	57 M	5 years	Toxic exposure to benzol; thymectomy and cortisone remission died of leukemia
XXI	35	1960	59 M	5 months	Improved by bone marrow transfusion
XXII	42	1960	69 M	1½ years	Diabetes mellitus developed after numerous transfusions; death from acute cardiac failure
XXIII	28	1961	18 M	4 years	Three remissions obtained with prednisone associated hypo-gamma-globulinemia

Table IV

Average peripheral blood differential counts at onset of anemia.

	Average per cent by cases	
	Reported in literature (14 cases)	Mayo Clinic series (18 cases)
Lymphocytes	33.2	32.8
Monocytes	6.0	7.8
Neutrophils	56.4	53.0
Eosinophils	4.4	4.3
Basophils	0.6	1.2

Bone marrow specimens were of normal overall cellularity on histologic sections, with selective virtual absence of all normoblastic forms in marrow smears. The ratio of myelocytes to erythroblasts in our cases at the time of initial examination ranged from 18.1 to 100.0 An increase in the number of lymphocytes was described in 5 reported cases (II, III V VII, and XII) In 11 of our cases, lymphocytosis was noted in the bone marrow (table II) Lymphoid cells with small, dark, pyknotic nuclei and scanty basophilic cytoplasm were seen in most of our cases. These unusual, peculiar cells, first described by CHALMERS AND BOHEIMER (8 9) differ in appearance from normal lymphocytes. They seem to be a characteristic finding in the marrow of patients with PRCA, but their origin is unknown. They may represent degenerating normoblasts which are unable to mature due to some defect or deficiency Since these cells (morphologically) resemble lymphocytes more than normoblasts, they have been counted as lymphocytes in bone marrow differential counts (table II) An increased number of eosinophils was mentioned in 6 cases from the literature (III V VII VIII, XIV and XVIII) and we noted this finding in 7 of our cases. Plasma cells and tissue basophils were increased in the marrow smears of several of our patients.

At the time of onset of disease, none of the patients in either series showed evidence of increased erythrocyte hemolysis. Results of radiochromium studies of erythrocyte survival are presented in table V Later in the course of the disease, hemolytic anemia developed in 4 instances (cases XII XVI XIX, and I) with generally favorable response to splenectomy and/or adrenal steroids. Studies of radioiron kinetics (table V) revealed a greatly prolonged disappearance rate of radioactive iron from the plasma and complete failure to incorporate iron into erythrocytes.

Table V

Rates of production and destruction of erythrocytes (studies with Fe^{59} and Cr^{51})

	Disappearance rate of plasma iron (Fe^{59}) (half-time) (Normal values: 60 to 120 minutes)	Rate of incorporation of Fe^{59} into erythrocytes (Normal values: 70 to 100% after 14 days)	Rate of erythrocyte destruction (Cr^{51} survival) (Normal values: Loss of less than 2.5% per day over 10 days)
Case XII		Less than 4% after 6 weeks	
Case XIII		5% after 19 days	
Case 2	More than 15 hours	0 after 14 days	
Case 11		31.4% after 10 days (on androgen therapy)	3.0% per day
Case 13	More than 3 hours	0 after 10 days	2.2% per day
Case 16	More than 15 hours	0 after 14 days	

Table VI

Toxic agents possibly involved as etiologic factors.

Toxic agent	Cases
Benzole, benzene hexachloride	X, XIX, XX, 4 10, 11
Sulfonamide	VIII XIV
Pentachlorophenol	13
Insecticides	9
Volatile solvent	6
Dry cleaning fluid	XV
Quinacrine	XI
Tolbutamide	I

In case XXIII, reported by LINK AND MURRAY (28) hypogammaglobulinemia was associated with PRCA. In case 1 of our series, a cryofibrinogen was demonstrated temporarily in the blood and at that time purpuric lesions appeared on the lower extremities.

In 14 of the 39 cases of both series, exposure to a toxic agent which may have played some part in the development of the disease was found (table VI) The assumption that the toxic factor might have played a significant role in causing the disease is strengthened by the fact that in two cases (VIII and 10) removal of the offending agent alone was followed by complete recovery from PRCA In case XIV reported by SEAMAN AND KOLER (44) the patient recovered from two separate episodes of PRCA after removal of a toxic factor but a third episode of PRCA developed several years later for which no toxic factor could be found In both instances in which sulfonamides were thought to be responsible for the onset of the disease, an allergic skin reaction was also present and was presumed to have been caused by hypersensitivity to the drug In all 6 patients exposed to compounds containing benzene hexachloride the exposure was long standing, extending over several months to

years. In case XX, described by SOUTTER AND EMERSON (48) exposure occurred 8 years prior to the onset of the disease, while in the other 5 cases the exposure was most prominent just prior to the onset of PRCA. In case 4 toxic hepatitis, which caused the patient's death, was present in association with PRCA and was presumed to be due to the same toxic exposure.

In our series of 16 cases, 8 patients are still alive at the time of this report. The duration of PRCA in the 8 patients who succumbed was 1 to 3½ years with an average of 2¼ years. The duration of the PRCA phase in our cases ranged from 5 months to 5 years. Because of the selective depression of erythropoiesis, prognosis seems to be better for patients with PRCA than for those suffering from aplastic anemia. Among 15 patients of both series who died as a result of PRCA (excluding those with terminal leukemia) cerebral hemorrhage due to terminal thrombocytopenia was the cause of death in only one patient, namely in case IV described by KARK (22)

As demonstrated in table VII death occurred most frequently in patients in whom exogenous hemochromatosis had developed. Transition into an acute, rapidly fatal leukemia occurred in 3 patients, one to three years after the onset of PRCA. Chronic granulocytic leukemia became apparent three years after onset of PRCA in one patient (case 16) who is still alive under treatment with busulfan and no longer requires blood transfusions (table VIII)

Table VII
Causes of death.

Cause of death	Cases
Hemosiderosis	III, V XIV XIX, 2, 8, 14
Leukemia	XX, 14 15
Transfusion reaction	VI, 2
Toxic hepatitis	4
Cardiac failure	XXII
Bacteremia	XIX
Terminal thrombocytopenia	IV
Accidental death	5
Cause not well documented	I II, VII, 1 11

Therapy

The only form of PRCA known to respond to specific therapy is the one recently described in association with kwashiorkor which can be cured by riboflavin (17 26 53) In 1953 FOY AND KONDI (16) described recovery from PRCA in a 46-year-old colored man

Table VIII
Course and outcome of leukemia in four cases.

Case	Type of leukemia	Duration of red cell aplasia	Duration of leukemia	Outcome
XX	Acut monocytic	4 ½ years	9 months	Dead
14	Acute	1 years	6 months	Dead
15	Acute	15 months	3 months	Dead
16	Chronic granulocytic	3 years	5 years	Alive

of Kenya by the administration of riboflavin This case most likely belonged to the afore mentioned special form of PRCA since other investigators have not reproduced the favorable effect of riboflavin therapy in American or European patients.

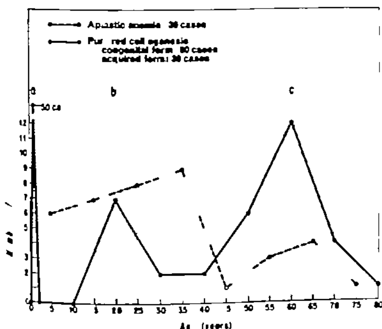


Fig. 1. Age distribution of patients ha ing aplastic anemia reported by SCOTT et al. (43) compared with that of patients ha ing pure red cell agenesis. *Peak a.* Congenital form according to DIAMOND AND BLACKFAN (11). *Peak b and c* Acquired form.

The disease has only rarely subsided spontaneousl In our series, a 67 year-old woman (case 7) who had required transfusions over a 5-year period, experienced a remission without any therapy in 1955 and is still alive at the time of this report. Similarly a 72 year-old man (case 13) recovered 9 months after the onset of the

illness and has been well for one year Two patients, case 3 from our series and case IX described by PALMÉN AND VAHLQUIST (37) recovered from congenital PRCA case IX recovered after the age of puberty while in case 3 remission occurred $2^1/_2$ years after transfusion of 35 pints of blood These latter two patients had a relapse of PRCA at the age of 21 and 15 years, respectively and therapeutic efforts were no longer of avail.

Removal of a toxic agent was followed by recovery of PRCA in several patients. In case VIII reported by STRAUSS (49) the patient recovered within 6 weeks after the use of sulfathiazole was discontinued. In our case 10 a 34-year-old woman with multiple allergies recovered from PRCA which had required 12 pints of blood, after cessation of repeated exposures to a vaporized insecticide containing Lindane (gamma isomer of hexachlorocyclohexane) In case XIV described by SEAMAN AND KOLER (44) the patient recovered from two separate episodes of PRCA after the use of a sulfonamide drug was stopped.

Several therapeutic approaches have been proposed in the past for PRCA with variable benefit. *Splenectomy* alone or combined with postoperative ACTH or adrenal steroid therapy was curative in three patients with superimposed hemolytic anemia (cases XII XVI, and XIX) These patients recovered from the hemolytic process as well as from PRCA. In patients with PRCA *alone*, splenectomy cured the anemia in case XIII described by LOEB et al (29) and together with cobalt therapy induced a remission in case XVIII reported by FOUNTAIN AND DALES (15) Splenectomy alone, however was performed without benefit in 5 patients (III IX 3 8, and 9) and oral administration of *cobalt* was ineffective in 5 others (XIX, XX, 1 3 and 5)

Adrenal steroids were administered to most of our patients and to several patients reported previously The overall results appear disappointing In case XX, described by SOUTTER AND EMERSON (48) thymectomy (in the absence of a thymoma) and adrenal steroids resulted in remission of longstanding PRCA. Acute monocytic leukemia developed later however and the patient died 9 months after thymectomy LINK AND MURRAY (28) obtained three separate remissions (case XXIII) with prednisone.

NICOLAU et al (35) induced a remission by the *transplantation of bone marrow* from a homologous donor in a 59-year-old man suffering from PRCA (case XXI)

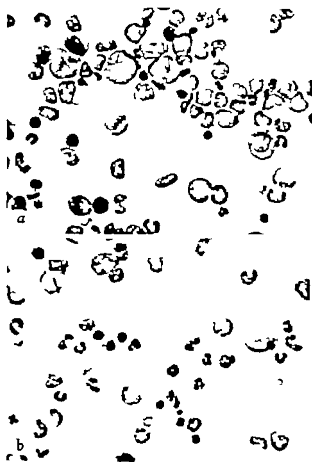


Fig. 2. Photomicrograph of bone marrow in case 11) prior to androgen therapy *b)* 4 weeks after androgen therapy Wright stain 500.

Long-term androgen therapy is known to affect the bone marrow by selective stimulation of erythropoiesis (24) SHAHIDI AND DIAMOND (45 46) have reported favorable response to androgen therapy in children with aplastic anemia and congenital hypoplastic anemia. We have found in the literature no reports of androgen therapy in adults with acquired PRCA without associated thymoma. While in our own experience short trials were inefficient in cases 1 3 14 and 16 continuous long-term administration of testosterone seemed to be beneficial in cases 11 and 12 One patient (case 12) has not required further blood transfusions since the sixth month of treatment and has maintained a hemoglobin concentration of 8 to 9 g/

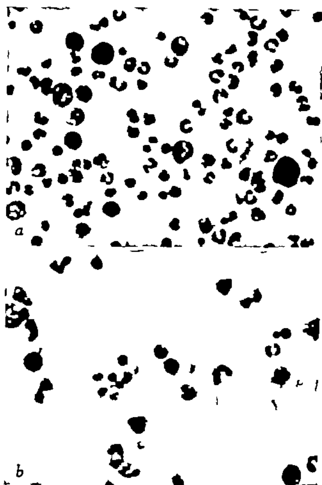


Fig 3. Photomicrograph of bone marrow in case 12) prior to androgen therapy b) 7 months after androgen therapy Wright stain 500.

100 ml for one year on androgen therapy The other patient (case 11) had required regular blood transfusions for 8 months and initially responded well to androgen therapy At the end of 6 weeks the patient had not required further transfusions. Reticulocytes rose from 0 1 to 2 6 / after 6 weeks of androgen therapy However the rate of incorporation of radioiron did not reach a normal level while testosterone was being administered, since only 31 / incorporation into erythrocytes was measured at the end of 10 days (table V) Normoblastic erythropoiesis, entirely lacking at first, could be demonstrated in the marrow smears during testosterone therapy (table II and fig 2a and 2b)

Figures 2a and 3a show photomicrographs of bone marrow smears of cases 11 and 12 prior to therapy. In figures 2b and 3b there is evidence of erythropoiesis in the marrow of these two patients, 4 weeks and 7 months following the administration of testosterone propionate, 100 mg intramuscularly three times weekly. Erythropoiesis, however, was still inadequate at that time (tables II and V). While one patient (case 12) obtained almost complete remission, the other patient (case 11) relapsed several months later and died.

Summary

Acquired pure red cell agenesis is an uncommon type of refractory anemia, characterized by the virtual absence of red cell precursors from an otherwise cellular marrow. The basic underlying defect is not known, but in some patients the disease may be initiated by a drug or by chemical exposure. In about 10% of the 59 cases reviewed, increased hemolysis developed, and about 10% of the cases terminated as leukemia. Males were affected about twice as frequently as females. The maximal incidence was after 50 years of age and during adolescence. No therapy has been uniformly successful. Some patients apparently have recovered after the administration of adrenal steroids or ACTH, and others have recovered after removal of an offending drug or chemical. From our limited experience it would appear that long-term androgen therapy may be helpful. In the presence of a superimposed hemolytic process, splenectomy alone or in combination with adrenal steroids seems indicated.

Résumé

L'agénésie érythrocytaire acquise est une forme rare des anémies réfractaires. Elle est caractérisée par une absence complète et persistante de l'érythropoïèse dans une moelle osseuse par ailleurs intacte et riche en cellules. L'étiologie de cette affection est inconnue. Dans près de la moitié des 59 cas étudiés, une exposition à des agents toxiques précédait le début de l'anémie. Dans environ 10% des cas un processus hémolytique fut être démontré dans des stades ultérieurs, et dans 10 autres % des cas on observe une leucémie terminale. La maladie commence presque toujours après la cinquantaine, à l'exception de quelques cas isolés entre 15 et 25 ans. Plusieurs cas furent guéris par traitement à la cortisone et à l'ACTH, ou encore par l'élimination de la substance toxique; cependant le plus souvent l'anémie fut totalement réfractaire au traitement. Selon notre expérience toutefois très limitée, un traitement aux androgènes de longue durée peut améliorer l'anémie, cependant la splénectomie et un traitement cortisonien sont préférables s'il y a une combinaison avec une anémie hémolytique.

Zusammenfassung

Die erworbene erythrozytäre Agenesis ist eine seltene Form von refraktärer Anämie. Sie ist gekennzeichnet durch langdauerndes, vollständiges Fehlen der Erythropoiese in einem sonst intakten, zellreichen Knochenmark. Die Ätiologie der Störung ist unbekannt. Bei etwa der Hälfte der durchgesehenen 39 Fälle kamen toxische Substanzen für die Auslösung der Krankheit in Frage. In zirka 10% der Fälle konnte in einem späteren Stadium eine gesteigerte Hämolyse nachgewiesen werden und zirka

10% der Fälle zeigten terminal eine Leukämie. Männer waren fast doppelt so häufig befallen wie Frauen. Die Krankheit trat fast immer nach dem 50. Lebensjahr auf, vereinzelt auch zwischen dem 15. und 25. Lebensjahr. Mehrere Patienten wurden durch Corticosteroide oder ACTH geheilt, andere durch die Eliminierung einer toxischen Noxe; meistens war die Anämie jedoch völlig therapierefraktär. Entsprechend unserer begrenzten Erfahrung kann langdauernde Androgentherapie die Anämie bessern. Bei Kombination mit einer hämolytischen Anämie ist die Splenektomie, allein oder in Verbindung mit einer Corticosteroid-Therapie, angezeigt.

References

1. ALDRIDGE, A. G. V. AND KIDD, P.: Primary red-cell aplasia. Brit. med. J. *1*: 729 (1953).
2. ALLEN, D. M. AND DIAMOND, L. K.: Congenital (erythroid) hypoplastic anemia. Cortisone treated. Amer. J. Dis. Child. *102*: 416 (1961).
3. ANDERSON, D. E.: Chronic aregenerative anaemia of the newborn. Med. J. Australia *1*: 573 (1952).
4. BAAR, H. S.: The life span of red blood corpuscles in erythronophthisis (including method for the estimation of faecal stercobilinogen). Acta haemat. *7*: 17 (1952).
5. [illegible], Z. AND KLEMENTOVA, V.: Erythrogenesis imperfecta. Čsl. pediat. *14*: 869 (1959).
6. BURGERT, E. O. Jr., KENNEDY, R. L. J. AND PEASE, GERTRUDE L.: Congenital hypoplastic anemia. Pediatrics *13*: 218 (1954).
7. CATHIE, I. A. B.: Erythrogenesis imperfecta. Arch. Dis. Childh. *25*: 313 (1950).
8. CHALMERS, J. N.: Pure red cell anemia in patients having tumors of the thymus. 6th Int. Congr. int. Soc. Hemat. 1956, p. 659 (Grune & Stratton, New York 1958).
9. CHALMERS, J. N. M. AND BOHEIMER, K.: Pure red-cell anaemia in patients with thymic tumours. Brit. med. J. *2*: 1514 (1954).
10. DIAMOND, L. K., ALLEN, D. M. AND MAGILL, F. B.: Congenital (erythroid) hypoplastic anemia. A 25 year study. Amer. J. Dis. Child. *102*: 403 (1961).
11. DIAMOND, L. K. AND BLACKFAN, K. D.: Hypoplastic anemia. Amer. J. Dis. Child. *56*: 464 (1938).
12. DONNELLY, M.: A case of primary red-cell aplasia. Brit. med. J. [illegible]: 438 (1953).
13. EISEMANN, G. AND DAMESHEK, W.: Splenectomy for "pure red-cell" hypoplastic (aregeneratory) anemia associated with autoimmune hemolytic disease. Report of a case. New Engl. J. Med. *251*: 1044 (1954).
14. ESSER, MARGRIT: Heilung eines Falles von congenitaler aplastischer Anämie Typus Benjamin. Ann. paediat. *157*: 366 (1941).
15. FOUNTAIN, J. R. AND DALES, M.: Pure red-cell aplasia successfully treated with cobalt. Lancet *i*: 541 (1955).
16. FOY, H. AND KONDI, ATHENA: A case of true red-cell aplastic anaemia successfully treated with riboflavin. J. Path. Bact. *65*: 559 (1953).
17. FOY, H., KONDI, ATHENA AND MACDOUGALL, LORNA: Pure red-cell aplasia in marasmus and kwashiorkor treated with riboflavine. Brit. med. J. *i*: 937 (1961).
18. GASSER, C.: Akute Erythroblastopenie. 10 Fälle aplastischer Erythroblastenkrisen mit Riesenproerythroblasten bei allergisch-toxischen Zustandsbildern. Helv. paediat. Acta *4*: 107 (1949).
19. HEILMEYER, L. UND BEGEMANN, H.: Die echte aplastische Anämie im engeren Sinne (Erythroblastophthise). In Hb. inn. Med., vol. 2, p. 882 (Springer, Berlin 1951).
20. HILL, J. M. AND HUNTER, R. B.: ACTH therapy in refractory anemias. Proc. 2nd clin. ACTH Conf., vol. 2, Therapeutics, p. 181 (Blakiston, New York 1951).
21. JOSEPH, H. W.: Discussion. Amer. J. Dis. Child. *56*: 466 (1938).

22. Kark, R. M. Two cases of aplastic anaemia. One with secondary haemochromatosis following 290 transfusions in nine years, the other with secondary carcinoma of the stomach. Guy Hosp. Rep. *87* 343 (1937)
23. Kaznelson, P Zur Entstehung der Blutplättchen. Verh. dtsch. Ges. inn. Med. *34* 557 (1922).
24. Kennedy B. J and Gilbertsen, A. S. Increased erythropoiesis induced by androgenic-hormone therapy New Engl. J Med. *256*: 719 (1957).
25. Kohler C. O. Congenital hypoplastic anemia Case report. J Pediat. *19*: 662 (1941).
26. Kondi, Athena; Mehta, S. H. and Foy H. Red-cell aplasia in marasmus and kwashiorkor Brit. med. J *1* 110 (1962)
27 Lemley, J T Anemia of the newborn A case report. J Pediat. *27* 263 (1945)
28. Lowe, J A. and Murray C. K. Erythrocyte aplasia and hypogammaglobulinemia Response to steroids in young adult. Ann. intern. Med. *55*: 831 (1961)
29. Loeb, V Jr Moore, C. V and Dubach, R. The physiologic evaluation and management of chronic bone marrow failure. Amer J Med. *15*: 499 (1953)
30. Lorenzo y de Ibarreta, J; Bidegain S. Barahona, L. and Garófalo, O Anemia arregenerativa del recien nacido (Tipo Josephs-Blackfan-Diamond) Arch. pediat. Uruguay *32*: 15 (1961)
31. Lupu, N. C. and Nicolau, G C. T Observations cliniques, hématologiques et histologiques sur un cas de leucoérythrophtisie. Sang *5*: 530 (1931)
32. MacFarlane, J W and Currie, J P Idiopathic aplastic anaemia A report of four cases. Edinburgh med. J *50*: 171 (1943)
33. Mackey R. An unusual case of aplastic anaemia with organ changes resembling haemochromatosis. Med. J Australia *1* 172 (1942)
34. Mosimann, S. und Rohr, K. «Aplastische Anämie» mit jahrelangem vollständigem Fehlen der Erythroblasten (Erythroblastophthise) Dtsch. Arch. klin. Med. *190* 117 (1943).
35. Nicolau, C. T Popescu, E.; Poppa, C. Apateanu, V Nedoara, S. Georgescu, G.; Butoianu, E. et Fotino, M. Erythrophtisie traitée par transfusion de moelle osseuse. Sang *31* 357 (1960)
36. O'Gorman Hughes, D W Hypoplastic anaemia in infancy and childhood. Erythroid hypoplasia. Arch. Dis. Child. *36*: 349 (1961)
37 Palmén, K. and Vahlquist B. Stationary hypoplastic anemia. Acta haemat. *4* 273 (1950).
38. Reeve, R. Two cases of idiopathic hypoplastic anemia in infants. Acta paediat. *28*: 304 (1940–1941).
39. Robson, T and Sweeney P J Chronic hypoplastic anaemia arising in infancy Arch. Dis. Child. *23*: 294 (1948)
40. Russell, L Hypoplastic congenital anemia. J Pediat. *20* 746 (1942).
41. Saxon, M. J Red cell aplasia Report of case and review of the literature. Arch. intern. Med. *94* 481 (1954).
42. Schaposnik, F and Vázquez, S. Aplasia eritrocítica. Sem. méd. esp. *117* 216 (1960).
43. Scott, J. L. Cartwright G. E. and Wintrobe, M. M. Acquired aplastic anemia An analysis of thirty-nine cases and review of the pertinent literature. Medicine *38*: 119 (1959)
44. Seaman, A. J and Koler, R. D. Acquired erythrocytic hypoplasia A recovery during cobalt therapy Report of two cases with review of the literature. Acta haemat. *9*: 153 (1953)
45. Shahidi, N. T and Diamond, L. K. Testosterone-induced remission in aplastic anemia. Amer J Dis. Child. *98*: 293 (1959)

46. Shahidi, N. T. and Diamond, L. K.: Testosterone-induced remission in aplastic anemia of both acquired and congenital types. Further observations in 24 cases. New Engl. J. Med. 264: 953 (1961).
47. Smith, C. H.: Hypoplastic and aplastic anemias of infancy and childhood; with consideration of the syndrome of nonhemolytic anemia of the newborn. J. Pediat. 43: 457 (1953).
48. Soutter, L. and Emerson, C. P.: Elective thymectomy in the treatment of aregenerative anemia associated with monocytic leukemia. Amer. J. Med. 28: 609 (1960).
49. Stralem, Anne M.: Erythrocyte aplasia following sulfathiazole. Amer. J. clin. Path. 13: 249 (1943).
50. Sutlorsa, N. e Bonvini, E.: L'anemia di Josephs-Diamond-Blackfan; Messa a punto sull'argomento e presentazione di un caso. Minerva pediat. 13: 1411 (1961).
51. Tsai, S. Y. and Levin, W. C.: Chronic erythrocytic hypoplasia in adults. Review of literature and report of a case. Amer. J. Med. 22: 322 (1957).
52. Vogel, P. and Bassen, F. A.: Sternal marrow of children in normal and in pathologic states. Amer. J. Dis. Child. 57: 245 (1939).
53. Walt, F.; Taylor, J. E. D.; Magill, F. B. and Nestadt, A.: Erythroid hypoplasia in kwashiorkor. Brit. med. J. i: 73 (1962).
54. Whitby, L. E. H. and Britton, C. J. C.: Disorders of the Blood. 5th ed. (Blakiston, Philadelphia 1946).

Authors' address: Drs. J. R. Schmid, J. M. Kiely and M. M. Hargraves, Mayo Clinic, 200 First Street South West, *Rochester*, Minn. 5590 (USA).

Acta haemat. 30 271–273 (1963)

Aus der II. Medizinischen Universitätsklinik (Vorstand Prof. Dr. K. Fellinger) und der II. Universitäts-Frauenklinik (Suppl. Leiter Doz. Dr. H. Braitenberg) Wien

Verhinderung schwerer Meno- und Metrorrhagien bei hämatologischen Erkrankungen durch einen Ovulationshemmer

Von H. Braunsteiner und H. Braitenberg

Schwere Meno- und Metrorrhagien im Verlaufe von hämatologischen Erkrankungen stellen ein schwieriges therapeutisches Problem dar. Bisher ist im wesentlichen nur eine symptomatische Substitutionstherapie mit Bluttransfusionen und Eisenpräparaten möglich gewesen. Die Indikation zur Röntgenkastration kommt bei vorübergehender Blutungsbereitschaft nicht in Frage und ist bei infausten Erkrankungen, wie etwa der akuten Leukämie, nicht anwendbar.

In den letzten Jahren ist durch die Entwicklung von Norsteroiden mit ausgeprägter ovulationshemmender Wirkung die Möglichkeit gegeben, eine künstliche, beliebig lang andauernde Amenorrhoe zu erzeugen. Auf therapeutischem Gebiet wurde daraus unseres Wissens lediglich zur Scheinschwangerschaftsbehandlung bei Sterilität (2) und zur Behandlung der Endometriose (1, 3, 4, 5, 6) Nutzen gezogen. Nach monatelanger Behandlung mit hochwirksamen Ovulationsunterdrückern (Anovlar, Enovid, Deluteval, Depot Provera, Lynestrenol, Norlutin) wurden keinerlei schädigende Nebenwirkungen gesehen. Nach Absetzen des Präparates haben die Patientinnen normal menstruiert und konzipiert.

Von diesen Erwägungen ausgehend haben wir uns zur Behandlung von hämatologischen Erkrankungen mit schweren Meno- und Metrorrhagien mit einem derartigen Präparat (4 mg Norethisteronazetat, 0,05 mg Aethinyl-oestradiol, «Anovlar Schering») entschlossen und geben einen vorläufigen Bericht über vier Patienten, wobei in jedem Fall das therapeutische Ziel erreicht wurde.

Kasuistik

Fall 1 23jährige Patientin mit akuter Leukose und allgemeiner schwerer Blutungsneigung, insbesondere mit ausgeprägten Metrorrhagien. Es bestand ein fast völliges Fehlen der Thrombozyten. Die Behandlung wurde mit höheren Corticoiddosen (50 bis 300 mg Prednisolon täglich) sowie mit 1 Tablette Anovlar täglich begonnen. Es kam zur Besserung des Allgemeinbefindens mit Anstieg des Hämoglobins, jedoch zu keiner hämatologischen Remission. Die Thrombozytenwerte blieben weiter unter 10 000/mm³. Anovlar wurde insgesamt 12 Wochen lang in der Dosierung von täglich 1 Tablette gegeben, während dieser Zeit kam es zu keinerlei Genitalblutungen, wobei gleichzeitig, insbesondere gegen Ende der Behandlungsperiode, ausgeprägte Schleimhautblutungen aus Nase und Zahnfleisch auftraten. Der Tod erfolgte nach etwa dreimonatiger Krankheitsdauer durch schwere Nasen- und Mundblutungen mit Aspirationspneumonie. Über den histologischen Befund der Genitalorgane wird gesondert berichtet.

Fall 2 32jährige Frau mit akuter Leukose. Es bestanden schwerste Metrorrhagien. Die Therapie wurde mit 6-Merkaptopurin, kleineren Dosen Prednisolon sowie 1 Tablette Anovlar täglich durchgeführt. Es kam zu einer teilweisen Remission mit Auftreten von 20 bis 25 % Granulozyten und 50 000 Thrombozyten/mm³. Anovlar wird seit insgesamt 21 Wochen gegeben. Es traten zweimal nächtliche Genitalblutungen auf, die auf vorübergehende Erhöhung der Dosis auf 2 bzw. 3 Tabletten Anovlar täglich sofort zum Stehen kamen. Die Patientin steht weiter in Behandlung der Klinik.

Fall 3. 20jährige Patientin mit Morbus Hodgkin, bei der es zu schwersten wochenlang anhaltenden Metrorrhagien bei Thrombopenie gekommen war. Eine Stillung dieser Blutung konnte durch keine Maßnahme erreicht werden. Unter Therapie mit 1 Tablette Anovlar täglich kam die Blutung nach 4 Tagen zum Stehen. Seither steht die Patientin unter Therapie mit 1 Tablette täglich, einmal war es notwendig, kurzdauernd wegen einer geringgradigen Durchbruchsblutung die Dosis auf 2 Tabletten zu erhöhen. Die bisherige Therapiedauer beträgt 23 Wochen, die Patientin steht unter Kontrolle der Klinik.

Fall 4 30jährige Patientin mit fast völligem Fehlen der Thrombozyten im Rahmen eines Morbus Gaucher. Die Splenektomie war unwirksam. Es traten schwerste Meno- und Metrorrhagien auf, die 3 bis 6 Transfusionen wöchentlich notwendig machten. Nach Therapie mit Anovlar kam es innerhalb von 5 Tagen zum Stehen der Blutung. Die Therapie wurde insgesamt 18 Wochen fortgesetzt, ohne daß es jemals zu einer Genitalblutung gekommen wäre, gleichzeitig bestanden jedoch immer wieder schwere Nasenblutungen. Auf einer gegen unseren Rat unternommenen Reise verstarb die Patientin an einer Gehirnblutung. Bei der Autopsie wurden die Genitalorgane nicht untersucht.

Diskussion

Aus den mitgeteilten Krankengeschichten geht der therapeutische Wert der Behandlung mit Ovulationshemmern bei jüngeren Patientinnen mit hämatologischen Erkrankungen, die an schweren Meno- und Metrorrhagien leiden, hervor. Nebenwirkungen wurden bisher nicht beobachtet. Keine der Patientinnen litt an Übelkeiten oder Kopfschmerzen. Geringgradig leichte Blutungen ließen sich durch Erhöhung der Dosis prompt unterdrücken. Nach Ferin (4) sind diese leichten Blutungen vorwiegend während der ersten Behandlungsmonate zu beobachten. Wir haben nur Patientinnen behandelt, die auf Grund ihres Krankheitszustandes nicht

konzeptionsfähig waren. Wie schon erwähnt interferiert die Behandlung nicht mit der Möglichkeit einer späteren Konzeption nach Absetzen des Mittels. Es tritt dann eine Blutung innerhalb einiger Wochen ein. Ovulation und Menstruation normalisieren sich. Über unsere weiteren Erfahrungen werden wir nach einer längeren Beobachtungsperiode berichten.

Zusammenfassung

Durch Dauerbehandlung mit einem hochwirksamen Ovulationsunterdrücker (Anovlar) wurde bei Patientinnen mit hämorrhagischer Diathese und schweren Meno- und Metrorrhagien ein völliges Sistieren der Genitalblutungen erreicht. Das Präparat führt nach allgemeiner Erfahrung zu keinen irreversiblen Veränderungen, so daß einer Konzeption nach Besserung des akut bedrohlichen Zustandes nichts im Wege steht. Nebenwirkungen wurden nicht beobachtet.

Summary

Continuous treatment with highly active inhibitor of ovulation (Anovlar) completely stopped genital bleeding in patients with haemorrhagic diathesis and severe menorrhagia and metrorrhagia. The treatment, according to general experience, does not produce any irreversible changes, so nothing need prevent conception once the acute emergency has been relieved. No side effects were observed.

Résumé

Le traitement, permanent vec un très puissant inhibiteur de l'ovulation (Anovlar) mène à un arrêt complet des hémorrhagies génitales chez des malades souffrant d une diathèse hémorragique et de sévères méno- et métrorragies. D après l'expérience générale le traitement ne provoque pas de changements irréversibles. Ainsi rien empêche une conception, aussitôt le danger éliminé. On ne pu observer de réactions secondaires.

Literatur

1. Andrews, M. C. Andrews, W. C. and Strauss, A. F. Effects of progestin-induced pseudopregnancy on endometriosis. Clinical and microscopic studies. Amer. J. Obstet. Gynec. *78*, 776 (1959)
2. Brattenberg, H. und Velika, L. Zur therapeutischen Verwendbarkeit der hormonellen Ovulationsblockade. Wien. med. Wschr. (im Druck)
3. Chalmers, J. A. Treatment of endometriosis with Anovlar. J. Obstet. Gynaec. brit. Emp. 69: 801 (1962)
4. Ferin, J. Treatment of endometriosis. 4th World Congr. Sterility Fertility, Rio de Janeiro, 8–15 August 1962
5. Kistner, R. W. The use of newer progestins in the treatment of endometriosis. Amer. J. Obstet. Gynec. *75*, 264 (1958). Conservative management of endometriosis. Lancet *i*, 179 (1959). The treatment of endometriosis by inducing pseudopregnancy with ovarian hormones. Fertil. Steril. *10*, 539 (1959). Infertility with endometriosis. A plan of therapy. Fertil. Steril. *13*, 237 (1962)
6. Rock, J.; Pincus, G. and Garcia, C. R. Use of some progestational 19-norsteroids in gynecology. Amer. J. Obstet. Gynec. *79*, 758 (1960)

Adresse der Autoren: Doz. Dr. H. Braunsteiner, II. Medizinische Univ.-Klinik, Garnisongasse,
Doz. Dr. H. Brattenberg, II. Univ.-Frauenklinik, Wien (Österreich).

Acta haemat. 30, 274–279 (1963)

Department of Clinical Medicine Medical School of Ribeirão Préto, State of São Paulo

Chromosome Abnormalities in Multiple Myeloma

By C. Bottura

The nature and pathogenesis of multiple myeloma are unknown. As it is generally regarded as a neoplastic disease chromosome abnormalities were to be expected. However the first cytogenetic studies performed on five cases of the disease failed to show any structural or numerical anomalies of the chromosomes (1, 9) Recently references were made to five additional cases, three of which had abnormal karyotypes (5 7 8)

As part of systematic studies on chromosomes of bone marrow cells in hemopoietic diseases, this communication deals with the chromosome findings in five cases of plasmocytome

Material and Methods

I all cases the chromosome analyses were carried out on bone-marrow cells obtained by sternal puncture and before any treatment was started. A direct method was used in which culture procedures are voided (3). A colchicine derivatis (Colcemid, CIBA) is injected intravenously and the marrow specimen collected tw hours afterward. After treatment with hypotonic solution, the suspension of cells is stained with the Feulgen reagent and squashed in the usual w y

Clinical Data

Case 1 J J 60-year-old white male, was admited to the hospital for pain in the lumbar spine and pelvis of 1 and half years duration, radiating into the legs with restriction of motion. On physical examination there was no lymph node enlargement or hepatosplenomegaly The skeletal roentgenograms revealed widespread demineralization of the pine pelvis and ribs, several punched out lesions in the upper ends of the femora, osteolytic lesions in the ribs and collapse of some ertebral bodies. R.B.C. 3,300 000 per mm^3 Hemoglobin 9,0 gm/100 ml W.B.C. 3,200 per mm^3 neutrophils 41 eosinophils 6 basophils 1 %, lymphocytes 43 and monocytes 9%). Sternal marrow biopsy plasma cell myeloma.

Case 2. A. C., 44-year-old white male entered the hospital complaining of pain in the left chest for 8 months. I recent months he had developed swelling at the level of the last three ribs on the lateral side of the left hest There as no hepatosplenomegaly or lymph node enlargement X-ray examination revealed destruction of the ninth

left rib, large osteolytic lesion in the first left rib, numerous small destructive lesions in other ribs and several osteolytic lesions in the pelvis. R.B.C. 3,700,000 per mm³ Hemoglobin 11,5 gm/100 ml W.B.C. 5,400 per mm³ (neutrophils 72% eosinophils 4%, basophils 2%, lymphocytes 17% and monocytes 5%). Sternal marrow biopsy plasma cell myeloma.

Case 3: E. C. S., 66-year-old white female was admitted t the hospital complaining of pain in the left elbow and forearm of four years duration. One year later she developed pain and numbness in the left foot, and pain in the left chest made worse by respiratory movements and coughing. Severe pain had developed in the lumbar spine in the last four months, and at the time of admission to the hospital she was completely bedridden. There had been progressive weight loss. No hepatosplenomegaly or lymphadenopathy were found. Bone X-ray revealed diffuse decalcification of the skull, ribs, spine and pelvis, without demonstrable focal lesions, and collapse of D12. R.B.C. 2,700,000 per mm³ Hemoglobin 8.9 gm/100 ml W.B.C. 6,500 per mm³ (neutrophils 51%, eosinophils 8%, basophils 1%, lymphocytes 34%, and monocytes 6%). Sternal marrow biopsy plasma cell myeloma.

Case 4 R. A. M., 61-year-old white male, entered the hospital with two years history of pain in the lower back, ribs, arms and legs which was relieved only by rest and analgesics. Six months before the initial symptoms he had suffered an injury to the left iliac crest with severe local pain which made him unable to walk for one month. He has been bedridden for the four last months because of increasing pain. Four days before admission to the hospital he developed complete motor and sensory paralysis of the lower extremities. He had anorexia, weakness and marked weight loss. In the hospital his state deteriorated rapidly and he died two months after admission. No liver spleen or lymph node enlargements. X-ray examination disclosed few osteolytic areas in the skull, mandible and both humeri and extensive lytic lesions in the spine, pelvis and ribs. R.B.C. 3,500,000 per mm³ Hemoglobin 11,2 gm/100 ml W.B.C. 6,300 per mm³ (neutrophils 56%, eosinophils 19%, basophils 0%, lymphocytes 20%, and monocytes 5%) Sternal and iliac marrow biopsies plasma cell myeloma. Post mortem examination revealed widespread myelomatous infiltration of the bones. Gonads and genitals were normal.

Case 5 A. F 42-year-old white male entered the hospital complaining of generalized weakness and pain in lower limbs of three months duration. During the course of the disease he had repeated episodes of fever and large nasal hemorrhage. On admission he was completely incapacited by severe bone pains and marked anemia. The liver was palpable 5 cm. below the right costal margin and the spleen tip was felt below the left costal margin. There was no lymphadenopathy The disease was rapidly progressive and he died 8 days after admission. X-ray examination revealed diffuse demineralization of the skeleton and numerous small osteolytic lesions in the skull, upper third of both humeri and in upper and lower ends of the femora. R.B.C. 1,200,000 per mm³ Hemoglobin 2.9 gm/100 ml. Platelets 50,000 per mm³ W.B.C. 5,500 per mm³ (promyelocytes 1%, metamyelocytes 2%, neutrophils 78% eosinophils 8%, basophils 1% lymphocytes 6%, monocytes 3% and plasmocytes 1%) 13 normoblasts per 100 W.B.C. Sternal and iliac marrow biopsies plasma cell myeloma. Postmortem examination showed multiple myeloma involving the bones, liver and spleen.

In table I are summarized the data on total proteins and their electrophoretic patterns and the values for serum calcium, phosphorus and alkaline and acid phosphatases.

Results and Comments

The five cases here reported present the complaints and bone roentgenologic changes commonly associated with multiple mye-

Table I

Blood chemistry in fiv cases of multiple myeloma.

Patient	Serum proteins, gm/100 ml: Total	Albumin	Alpha₁	Alpha₂	Beta	M	Gamma	Serum calcium [illegible]	Serum phosphorus [illegible]	Alkaline phosphatase [illegible]	Acid phosphatase [illegible]
J J	7 7	2.71	0.50	0.55	0.7?	2.43	0.99	10.3	3.5	2.3	
A. C.	9 6	2.71	0.34	0.80	4.86		0.87	12.1	4.4	4 4	
E. C. S.	9.0	1.96	0.28	0.99	0.71		5.04	7.3	3.0	2.6	
R. A. M.	7 1	3.70	0.32	1.66	0.62		0.77	11.9	3.1	3.4	0.3
A. F	8.2	1.20	0.09	0.11	0.34	–	6.43	8.8	3.9	4.7	1.3

Table II

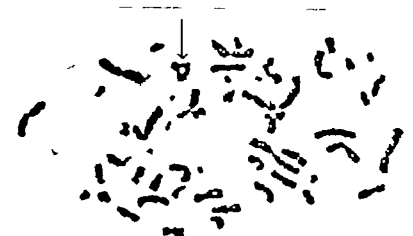
Myeloma types and chromosome counts on bone-marrow cells.

Patient	Sex	Age	Myeloma type	Total Cells Counted	Number of chromosomes: 44	45	46	51	92
J. J	M	60	M	50	1		48		1
A. C.	M	44	Beta	50	2		48		
E. C. S.	F	66	Gamma	25			25		
R. A. M.	M	61	Alpha₂	71		47	23		1
A. F	M	42	Gamma	33			34	1	

loma. The values for serum calcium, inorganic phosphorus and alkaline and acid phosphatases provided evidence against the presence of other destructive bone diseases, and the myeloma cell proliferation found in bone marrow aspirations confirmed the diagnosis of plasma cell myeloma. In cases 4 and 5 the diagnosis was further confirmed at postmortem examination.

The results of chromosome counts are shown in table II No chromosome abnormalities could be detected in the first three cases showing the electrophoretic patterns of the types beta, gamma and M (abnormal fraction with mobility between beta and gamma) However there was no evidence that the metaphases analysed were actually mitotic myeloma cells. Besides minor aberrations could be present and not be detected morphologically by the method used.

In patient A. F., a gamma type myeloma only one cell with abnormal karyotype was observed (fig 1) In spite of the poor quality of the preparation, at least 51 chromosomes plus 4 or 5 fragments could be discerned In addition one chromosome showed a complex configuration which was difficult to interpret. Like two cases reported by Lewis and MacTaggart (7) this patient was also in the terminal stage of the disease at the time of investigation.



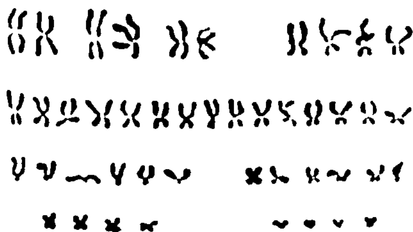


Fig. 1 Metaphase of bone marrow cell from patient A. F At least 51 chromosomes and 4 or 5 fragments can be discerned. Complex chromosome aberration is arrowed.
Fig. 2. Karyotype of metaphase cell from patient R. A. M. showing 45 chromosomes. One small acrocentric autosome is missing.

Although no pathogenetic significance can be assigned to an abnormality observed in one single cell, it seems very plausible that this was a neoplastic cell.

A quite different picture was shown by patient R A. M Counting disclosed two clones of cells. One with 46 chromosomes

had an apparently normal karyotype and these were considered as normal bone marrow cells in mitosis. The other clone with 45 chromosomes, showed a constant abnormality consisting of the absence of one small acrocentric autosome either 21 or 22 (fig. 2). The Y chromosome appeared to be present and an XY/XO mosaicism prior to the initiation of the disease is unlikely. Normal gonads were seen at postmortem examination and the patient was the father of eleven children. Unfortunately no skin or blood culture was performed to settle this.

The abnormality observed in this patient might have arisen during the course of the disease. In this case it would not be of primary etiological significance. However if detected in other cases of the disease, this finding would be of interest on several counts. Firstly it suggests the genetic nature of multiple myeloma. Secondly it would be another hemopoietic disease in which a small acrocentric autosome is involved the other one being chronic myeloid leukaemia. Finally, there seems to be an increased tendency towards acute leukaemia in mongolism which is a trisomic state for one small autosome. These observations suggest that a pathogenetic interrelationship may occur in these apparently so different diseases.

As in the case of acute leukaemia chromosome abnormalities in multiple myeloma have been inconstant and no two published examples have been the same. At all events there is a clear distinction between the diversity of the findings in myeloma and the relatively consistent pattern in the related macroglobulinaemia of WALDENSTRÖM (2 4 6 8).

Summary

Chromosome analyses were carried out on bone-marrow cells in five untreated cases of multiple myeloma. No chromosome abnormality was detected in three. In one patient only one out of 33 metaphases analysed showed an abnormal karyotype. In the last case the majority of cells had 45 chromosomes and the missing one was a small acrocentric autosome. The pathogenetic significance of these findings is discussed.

Résumé

Dans 5 cas de plasmocytes non traités on examina les chromosomes dans des cellules de la moelle osseuse. On ne trouva rien d'anormal dans 3 cas. Dans un cas, une des 33 métaphases étudiées présentait un caryotype anormal. Chez le dernier cas la majeure partie des cellules avait 45 chromosomes, et il manquait un petit autosome acrocentrique. La signification pathogénique de ces résultats est discutée.

Zusammenfassung

Bei 5 unbehandelten Patienten mit Plasmocytom wurden Chromosomen-Analysen an Knochenmarkzellen vorgenommen. In 3 Fällen fanden sich keine Anomalien. In einem Fall zeigte eine von 35 untersuchten Metaphasen einen abnormen Karyotyp. Beim letzten Fall wies die Mehrzahl der Zellen nur 45 Chromosomen auf, wobei ein kleines akrozentrisches Autosom fehlte. Die pathogenetische Bedeutung dieser Befunde wird besprochen.

References

1. Baikie, A. G.; Court Brown, W. M.; Jacobs, P. A. and Milne, J. S.: Chromosome studies in human leukaemia. Lancet *ii:* 425–428 (1959).
2. Benirschke, K.; Brownhill, L. and Ebaugh, F. G.: Chromosomal abnormalities in Waldenström's macroglobulinaemia. Lancet *i:* 594–595 (1962).
3. Bottura, C. and Ferrari, I.: A simplified method for the study of chromosomes in man. Nature *186:* 904–905 (1960).
4. Bottura, C.; Ferrari, I. and Veiga, A. A.: Chromosome abnormalities in Waldenström's macroglobulinaemia. Lancet *i:* 1170 (1961).
5. Castoldi, G. L.; Ricci, N.; Punturieri, E. and Bosi, L.: Chromosomal imbalance in plasmocytoma. Lancet *i:* 829 (1963).
6. German, J. L.; Biro, C. E. and Bearn, A. G.: Chromosomal abnormalities in Waldenström's macroglobulinaemia. Lancet *ii:* 48 (1961).
7. Lewis, F. J. W. and MacTaggart, M.: Chromosome counts in myeloma. Human Chromos. Newsl. *8:* 20–21 (1962).
8. Pfeiffer, R. A.; Kosenow, W. and Bäumer, A.: Chromosomenuntersuchungen an Blutzellen eines Patienten mit Makroglobulinämie Waldenström. Klin. Wschr. *40:* 342–344 (1962).
9. Richmond, H. G.; Ohnuki, Y. and Pomerat, C. M.: Multiple myeloma—an "in vitro" study. Brit. J. Cancer *15:* 692–700 (1961).

Author's address: Dr. C. Bottura, Dept. of Clinical Medicine, Medical School of *Ribeirão Preto*, State of São Paulo (Brazil).

Acta haemat. 30: 280–287 (1963).

From the Institute of Clinical Medicine, University of Freiburg i. Br. (Director: Prof. L. Heilmeyer)
Department of Immunopathology (Prof. H. Schubothe)

Studies on the Antigens of Human Red Cell Ghosts

Differences in Antigenic Specificity of Human Normal Adult, Hereditary Spherocytosis and Auto-Immune Haemolytic Anaemia S Protein

By Nicoletta Vulpis *

In the previous paper (5) data were presented which suggest that normal adult and new born S proteins differ in their serologic behavior and that this can be explained on the basis of differences in their antigenic specificity

The present investigation was undertaken to determine whether immunologically distinct varieties of S protein are associated with haemolytic syndromes, whether they are due to intrinsic defects of the erythrocyte or to mechanisms extrinsic to the cell. With the aid of rabbit antisera an analysis was made of the antigenic components in two S protein preparations, the former (SEHS) obtained from a patient with hereditary spherocytosis and the latter (SEAIH) from a case of auto-immune haemolytic anaemia of the warm antibody type.

Methods

Preparation of S protein solutions: The SEHS and SEAIH solutions were prepared according to the method of Moskowitz et al. (2) Two specimens of normal S protein (SEN_1 and SEN_2) were taken from two normal healthy donors and used for comparative study The nitrogen contents of all samples of S protein were determined by micro-Kjeldahl method thereafter they were brought to the same nitrogen content (30 mg%) by suitably diluting with distilled water

Preparation of antisera. Two groups of two young rabbits weighing initially approximately 2.4–2.8 kg were used. They were immunized with total of 18.0 mg. of SEHS and SEAIH per rabbit respectively by the method previously outlined (5). One of the two rabbits injected with SEHS died during immunization. When sufficiently high titers were obtained, the animals were bled either by cardiac puncture, or from ear vein, the serum removed from the clotted blood and stored in sterile screw [illegible] kept at −70° C until used. They were usually absorbed free of serum proteins and large globin antibodies by adding 0.30 ml human serum or crystalline oxyhaemoglobin

* This work was carried out while the author was NATO grantee in Germany.

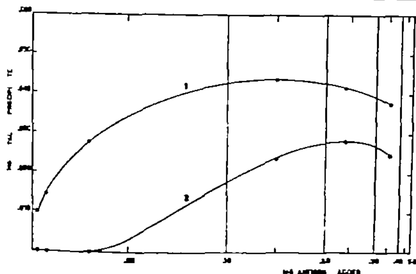


Fig. 1. Quantitative course of the precipitin reaction of rabbit anti-SEN_1 serum with homologous and heterologous antigen. Curve 1 refers to anti-SEN_1 + SEN_1, curve 2 to anti-SEN_1 + SEHS.

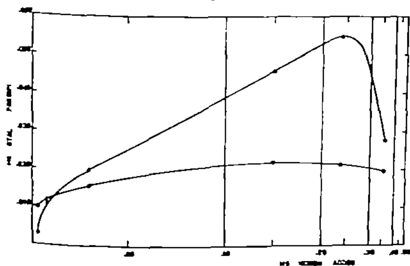


Fig. 2. Reaction of rabbit anti-SEHS serum and SEN_2. Curve 1 is for the homologous reaction, curve 2 for the cross reaction.

solution per ml antiserum. The serum and haemoglobin concentration was 5.03 and 1.05 g/100 ml respectively.

The antisera will be referred as anti-SEHS serum when the immunization was carried out with SEHS and $anti_1$- and $anti_2$-SEAIH when SEAIH served as antigen. An anti-SEN serum was employed as control.

Immunochemical techniques The procedures used for the quantitative precipitin test (1) OUCHTERLONY double diffusion in agar gel (6) and immuno-electrophoresis (3) have been described in earlier communications (4 5)

Results

Comparison of normal adult and hereditary spherocytosis S protein. The anti-SEHS serum gave more precipitate with SEHS than with SEN_1 The data of such serum are recorded in fig 1 With anti-SEN_1 serum it was also found that more antibody could be precipitated by the homologous antigen than by SEHS (fig 2) The same results were obtained with another preparation of normal adult S protein (SEN_2) (fig 3) This may be due either to a common identical antigen or to a chemically related but not identical antigen.

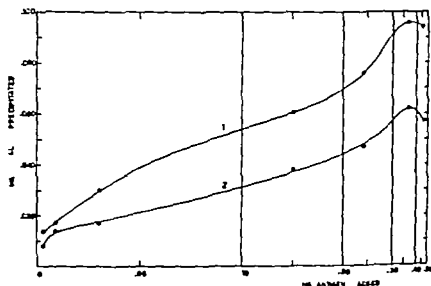


Fig 3. Quantitative immunochemical curves showing cross reaction of SEN_2 with homologous anti-SEN_2 (curve 1) and heterologous anti-SEHS immune sera (curve 2).

The results obtained in the OUCHTERLONY agar-diffusion experiments clearly showed that no difference was present in the antigenic specificity of normal adult and hereditary spherocytosis S proteins. In fact, the sera anti-SEN_1 and anti-SEHS formed with SEN_1 as well as with SEHS three precipitin bands forming fusion patterns as shown in fig 4 This implies that the two antigens being

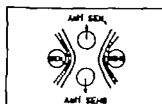


Fig. 4. Comparative double-diffusion analysis of SEN and SEHS by means of anti-SEN_1 and anti-SEHS immune sera. SEHS shows three precipitates that identify with the three lines obtained with SEN_1.

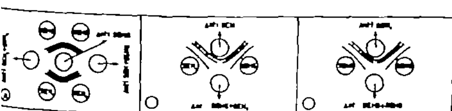


Fig. 5. Absorption of anti-SEN_1 ( ) and anti-SEHS (b,) with SEN_1 and SEHS causes disappearing of the precipitin bands.

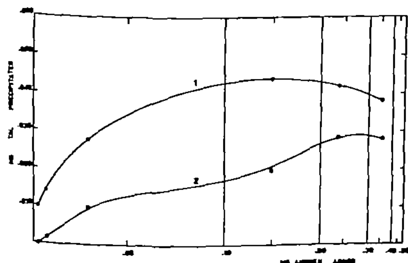


Fig. 6. Quantitative course of the precipitin reaction of rabbit anti-SEN_1 serum with homologous and heterologous antigen. Curve 1 refers to anti-SEN + SEN, curve 2 to anti-SEN_1 + SEALH.

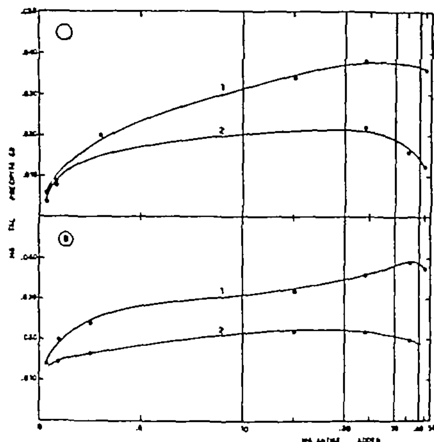


Fig 7 Reaction of rabbit anti$_1$-SEAIH ( ) and anti$_2$-SEAIH (b) with SEAIH and SEN Curves 1 are for the homologous reaction, curves 2 for the cross reaction.

compared are *identical* serologically A sample of each serum was absorbed both with SEN$_1$ and SEHS at the ratio of 0 06 mg S protein to 1 0 ml of serum. After 4 hours of incubation at 38 C the precipitate was centrifuged off The supernatants were then reacted against SEN$_1$ and SEHS and failed to give precipitation bands (fig 5a, b c)

Comparison of normal adult and auto-immune haemolytic anaemia S protein In contrast to the absence of antigenic specificity between normal adult and hereditary spherocytosis S protein, a difference in specificity was found to exist between normal adult and auto-immuno haemolytic S protein. Quantitative data on the reaction of SEN$_1$ and SEAIH with homologous and heterologous antisera are plotted in fig 6 and 7 The results there presented demonstrate

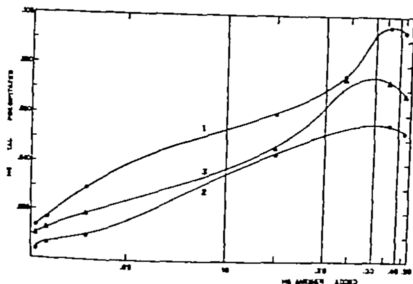


Fig. 8. Quantitative immunochemical curves showing cross reaction of SEN with homologous anti-SEN_1 serum (curve 1) and heterologous anti-SEAIH and anti-SEAIH immune sera (curve 2 and 3).

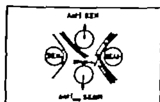


Fig. 9. Double-diffusion diagnostic test simultaneously comparing reaction of anti-SEN_1 and anti-SEAIH immune sera with SEN_1 and SEAIH. With reference to SEN_1, anti-SEAIH forms single precipitin band fusing with similar band produced by anti-SEN_1. In regard to SEAIH the two immunosera form three precipitin bands, one of which gives reaction of partial interference.

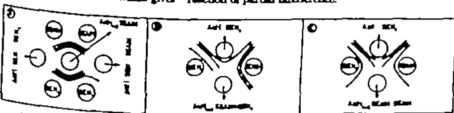


Fig. 10. Comparative double-diffusion analysis of SEN_1 and SEAIH, using absorbed anti-SEN_1 and anti-SEAIH immune sera) After absorption with SEN anti-SEN_1 does not give any precipitin reaction; after absorption with SEAIH the same immune serum produces only a single precipitin band with SEN_1 b) After absorption with SEN_1, anti-SEAIH produces two precipitates with SEAIH.) After absorption with SEAIH it gives only precipitin band with SEN

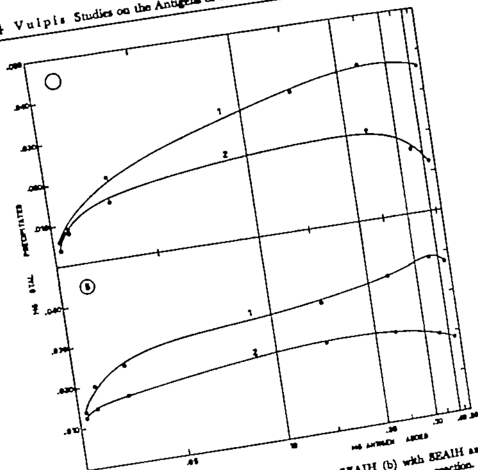


Fig 7 Reaction of rabbit $anti_1$-SEAIH (a) and $anti_2$-SEAIH (b) with SEAIH and SEN Curves 1 are for the homologous reaction, curves 2 for the cross reaction.

compared are *identical* serologically A sample of each serum was absorbed both with SEN_1 and SEHS at the ratio of 0.06 mg S protein to 1 0 ml of serum. After 4 hours of incubation at 38 C the precipitate was centrifuged off The supernatants were then reacted against SEN_1 and SEHS and failed to give precipitation bands (fig 5a, b c)

Comparison of normal adult and auto-immune haemolytic anaemia S protein. In contrast to the absence of antigenic specificity between normal adult and hereditary spherocytosis S protein, a difference in specificity was found to exist between normal adult and auto-immuno haemolytic S protein. Quantitative data on the reaction of SEN_1 and SEAIH with homologous and heterologous antisera are plotted in fig 6 and 7 The results there presented demonstrate

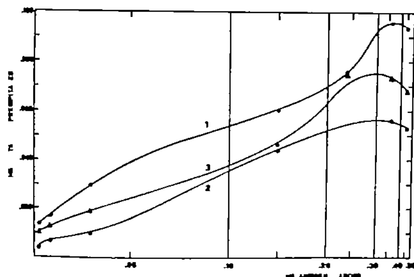


Fig. 8. Quantitative immunochemical curves showing cross reaction of SEN with homologous anti-SEN_1 serum (curve 1) and heterologous $anti_1$-SEAIH and $anti_2$-SEAIH immune sera (curve 2 and 3)

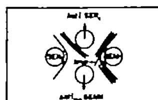


Fig. 9. Double-diffusion diagnostic test simultaneously comparing reaction of anti-SEN and $anti_{1+2}$-SEAIH immune sera with SEN_1 and SEAIH. With reference to SEN $anti_{1+2}$-SEAIH forms single precipitin band fusing with similar band produced by anti-SEN_1. In regard to SEAIH the two immunsera form three precipitin bands, one of which gives reaction of partial interference.

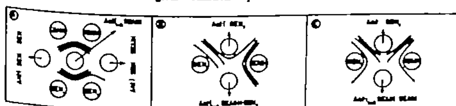


Fig. 10. Comparative double-diffusion analysis of SEN_1 and SEAIH, using absorbed anti-SEN_1 and $anti_{1+2}$-SEAIH immune sera:) After absorption with SEN anti-SEN_1 does not give any precipitin reaction; after absorption with SEAIH the same immune serum produces only single precipitin band with SEN_1 b) After absorption with SEN_1, $anti_{1+2}$-SEAIH produces two precipitates with SEAIH. c) After absorption with SEAIH it gives only a precipitin band with SEN_1.

their immunochemical properties. Results suggest that no differences exist between the antibodies developed against the normal adult and hereditary spherocytosis S proteins but did confirm the presence of specific precipitins for the auto-immune haemolytic anaemia S protein.

Résumé

Les propriétés immuno-chimiques des antisérums contre les protéines S de sphérocytoses héréditaires et d'anémies auto-immuno-hémolytiques furent comparées. On ne trouva aucune différence entre les anticorps contre les protéines S normales de l'adulte et les anticorps contre les protéines S de la sphérocytose héréditaire. Par contre il existe des précipitines spécifiques pour les protéines S des anémies auto-immuno-hémolytiques.

Zusammenfassung

Es wurde untersucht, ob Antiseren gegen S-Proteine von hereditärer Sphärocytose und von autoimmun-hämolytischen Anämien sich in ihren immunochemischen Eigenschaften unterscheiden. Aus den Ergebnissen geht hervor, daß keine Unterschiede bestehen zwischen Antikörpern gegen normales S-Protein des Erwachsenen und solchen gegen S-Proteine hereditärer Sphärocytosen, daß jedoch spezifische Präzipitine für die S-Proteine autoimmun-hämolytischer Anämien vorkommen.

References

1. Grabar, P.: La technique immuno-chimique quantitative et son emploi en immunologie et en chimie. Techniques de Laboratoire (Masson, Paris 1954).
2. Moskowitz, M.; Dandliker, W. B.; Calvin, M. and Evans, R. S.: Studies on the antigens of human red cells. I. The separation from human erythrocytes of a water soluble fraction containing the Rh, A and B factors. J. Immunol. 65: 383–392 (1950).
3. Scheidegger, J. J.: Une micro-méthode de l'immuno-électrophorèse. Int. Arch. Allergy 7: 103–110 (1955).
4. Vulpis, N. and Vulpis, G.: Études sur la constitution antigénique du stroma normal par des méthodes de précipitation spécifique en milieu gélifié et d'immuno-électrophorèse. Constitution antigénique de la protéine S. N. Rev. franç. Hémat. 2: 535–550 (1962).
5. Vulpis, N.: Studies on the antigens of human red cell ghosts. Differences in the S proteins of the adult and newborn. Acta haemat. 30: 229–235 (1963).
6. Wadsworth, C.: A microplate technique employing a gel chamber compared with other micro- and macroplate techniques for immune-diffusion. Int. Arch. Allergy 21: 131–137 (1962).

Author's address: Dr. [illegible] Vulpis, Via de [illegible] 23, [illegible] (Italy).

Acta haemat. 30: 288–304 (1963).

Aus der Medizinischen Klinik der Universität Tübingen
(Direktor: Prof. Dr. H. E. Bock)

Der fluoreszenzserologische Nachweis Leukocyten-spezifischer Antikörper*

Von W. Hartl

Das von Coons und Kaplan (16, 17) angegebene Verfahren, menschliches γ-Globulin *mit Hilfe Fluorescein-markierter Antiseren fluoreszenzoptisch histochemisch nachzuweisen*, hat im Zuge einer vielseitigen Anwendung auf zahlreichen Gebieten der modernen Immunologie zum Verständnis immunbiologischer Vorgänge beitragen können. Obschon man sich auch auf dem immunhämatologischen Sektor bereits seit längerer Zeit mit den Möglichkeiten eines fluoreszenzserologischen Antikörpernachweises befaßt hat, liegen zur Serologie der Leukocyten gesicherte, methodische Erfahrungen bisher kaum vor. Für die Serodiagnostik des Lupus erythematodes disseminatus wurde ein fluoreszenzserologischer Anti-Humanglobulin-Test an Leukocytenkernen und anderen Substraten humanen und tierischen Ursprungs mehrfach als empfindliche und zuverlässige Methode beschrieben (1, 4, 11, 12, 26, 60). Die klinische Verwendbarkeit dieses Tests zum Nachweis Leukocyten-spezifischer Iso- bzw. Autoantikörper ist dagegen bis *heute nur in sehr beschränktem Umfange untersucht worden* (11, 12, 24, 37, 50). Vor allem technischen Schwierigkeiten bei der Herstellung geeigneter Leukocytenpräparationen (31) und brauchbarer Antiseren (27, 40, 54, 56) sowie der vielfach ungenügenden Beherrschung der gerade bei den Leukocyten so störenden, unspezifischen Fluoreszenzeffekte (42, 60) dürfte es zuzuschreiben sein, daß die in anderen Zweigen der Immunologie so bewährte Methodik im Bereich der Leukocytenserologie bisher keine breitere Anwendung gefunden hat.

* Auszugsweise vorgetragen am 9. Kongreß der Europäischen Gesellschaft für Haematologie in Lissabon, 26.–31. August 1963.

Es war das Ziel der vorliegenden Versuchsreihe, zunächst möglichst exakt definierte, technische Voraussetzungen für einen fluoreszenzserologischen Anti Humanglobulin-Test (F AHG-T) an menschlichen Leukozyten zu schaffen der reproduzierbare Ergebnisse erwarten läßt. Der Test war dann im Hinblick auf seine Aussagefähigkeit als Nachweismethode Leukocyten-spezifischer Antikörper zu prüfen und hinsichtlich Empfindlichkeit und Spezifität mit dem in der Praxis bereits bewährten Leukocytenagglutinationstest (19 22 31 35 45 53) sowie dem Anti Humanglobulin-Konsumptionstest (45 49 57) in Vergleich zu setzen.

Methodik

Um möglichst übersichtliche Versuchsbedingungen zu erreichen, wurde der F-AHG-T zunächst ausschließlich im Sinne eines indirekten Testes, d. h. an Leukocyten einer beschränkten Zahl von Spendern der Blutgruppe 0 mit möglichst vielen Patientenseren, durchgeführt.

Untersuchungsmaterial

Die einzelnen, in die Untersuchungsreihe einbezogenen Patientenseren wurden folgendermaßen aufgeteilt:

1 Kontrollpersonen: 100, ohne Berücksichtigung der klinischen Diagnose ausgewählte Seren des allgemeinen Krankengutes der Klinik, wobei hämatologische Erkrankungen, allergische Affektionen im weitesten Sinne, Rheumatismus- und Kollagenosefälle sowie sämtliche Patienten, die Bluttransfusionen erhalten hatten, ausgeschlossen wurden.

2. Polytransfundierte: 19 Patienten, die zwischen 8 und 140 Bluttransfusionen erhalten hatten und teilweise (insgesamt 7) unter der Transfusionsbehandlung Unverträglichkeitserscheinungen zeigten, die bereits klinisch den Verdacht auf das Vorliegen leukocytärer Antikörper nahelegten (9). Im einzelnen handelte es sich um folgende Grundleiden: Akute Leukose (1) unklares »Leukämoid« (1), chronische Myelose (8) chronische Lymphadenose (1) aplastische Anaemie (4) Panzytopenie (1), Plasmocytom (1), Morbus Hodgkin (1) Colitis ulcerosa (1) Untersucht wurden ferner weitere 6 Patientenseren ohne Kenntnis der genauen Diagnose, die auf Grund multipler Bluttransfusionen einen stark positiven Leukocytenagglutinationstest (LAT) aufwiesen und von verschiedenen, auswärtigen Blutspendezentren in dankenswerter Weise zur Verfügung gestellt worden waren.

3. Lupus erythematodes disseminatus. 6 Fälle, bei denen die Diagnose klinisch und durch Nachweis des LE-Zellphänomens gesichert werden konnte. Bei 3 weiteren Patienten bestand auf Grund des klinischen Bildes der starke Verdacht auf das Vorliegen eines LED der schlüssige Beweis konnte jedoch nicht erbracht werden.

4. Agranulocytosen und Immunleukopenien. Hier handelte es sich um 6 Patienten mit klinisch typischem Bild der Agranulocytose strenger Definition (6, 29). Das auslösende, medikamentöse Agens war nur in einem Falle einer Neotchen-bedingten Immun-Leukopenie an Hand der Anamnese zu eruieren, in den anderen Fällen mußte die Frage nach dem Allergen offen bleiben.

Herrn Prof. Dr. J. J. van Loghem, Amsterdam, sowie Herrn Prof. Dr. J. Dausset, Paris, sei für die Unterstützung dieser Untersuchungen an dieser Stelle herzlich gedankt.

5. Varia: 2 Kranke mit dem Vollbild der «pluriorifiziellen Ektodermose» (Stevens-Johnson-Syndrom) ohne klinische Hinweise auf eine Störung der Leukopoese waren durch positive Befunde im Rahmen der Untersuchungsreihe aufgefallen. Untersucht wurden ferner 2 Fälle mit sogenanntem «Kälte-Agglutinin-Syndrom», bei denen sich hochtitrige Kryohämagglutinine nachweisen ließen. Einer dieser Kranken bot auch klinisch ein ausgeprägtes Raynaud-Syndrom bei Kälteexposition (32). Auch er war unter der Beobachtung nie leukopenisch. Ein letzter Kranker litt an einer fortgeschrittenen Dermatomyositis, auch hier lagen unauffällige Blutbildverhältnisse und keine sonstigen abnormen serologischen Befunde vor.

Technik des fluoreszenzserologischen AHG-Testes

Leukocytenpräparation. Wie zur Technik des LAT kürzlich ausführlich beschrieben (31) wurden Leukocyten von Spendern der Blutgruppe 0 Rh, die eine normale Blutsenkungsreaktion bei unauffälligem Blutbild und Serumelektrophoresebefund aufwiesen, durch Ammoniumchloridhämolyse aus EDTA-Vollblut angereichert, 3mal in einer Glukose-haltigen, physiologischen Salzlösung nach Sellomaier (cit. 59) gewaschen und schließlich in dünnem Ausstrich auf sorgfältig vorgereinigte Objektträger gebracht. Die lufttrockenen Präparate kamen zur Fixation 30 Min. in wasserfreies Aceton, das durch Zusatz von fester Kohlensäure auf etwa —70° C abgekühlt worden war. Aufbewahrung der fixierten Präparate bei +4° C im Kühlschrank, jedoch nie länger als 24 Stunden bis zur endgültigen Verwendung.

Antiseren. Verwendet wurden ausschließlich kommerzielle Antiseren der Firma Sylvana Company, Orange, New Jersey und zwar: a) im direkten Test (16, 17) 1. Anti-Humanglobulin vom Kaninchen (Vollserum) Fluorescein konjugiert. 2. Anti-Humanglobulin vom Kaninchen (Globulinfraktion) Fluorescein konjugiert. – b) zum indirekten Test 3. Anti-Humanglobulin vom Kaninchen (Globulinfraktion). 4. Anti-Kaninchen-Globulin vom Schaf (Globulinfraktion) Fluorescein konjugiert.

Zur Minderung unspezifischer Fluorochromierungseffekte wurden die Fluorescein-markierten Antiseren vor ihrer Verwendung zunächst mit Organtrockenpulver aus Schweineleber und anschließend zweimal mit getrockneten, plasmafrei gewaschenen, menschlichen Leukocyten (20 mg/ml) vorbehandelt. Dies geschah jeweils durch Inkubation über 30 Min. bei 37° C sowie nachfolgende Aufbewahrung im Eisschrank für 12 Stunden bei +4° C. Zur Entfernung störender Zellreste wurden die Antiseren schließlich hochtourig bei +4° C auszentrifugiert.

Vor ihrer Verwendung wurden die AHG-Seren an Hand einer mit inkompletten Anti-D sensibilisierten Erythrocytensuspension austitriert. Die Titer lagen für das konjugierte Anti-Humanglobulin vom Kaninchen bei 1:32 (Vollserum) bzw. 1:1280 (Globulinfraktion). Titer des unmarkierten AHG-Globulins vom Kaninchen 1:5000.

Eigentlicher Test. Die fixierten Leukocytenpräparate kamen zunächst 15 Min. in Phosphat-gepufferte, physiologische Kochsalzlösung (pH 7,2). Auf etwa 0,5 cm. im Durchmesser große, vorgezeichnete Areale, die beim Abwischen der Objektträger ausgespart und feucht blieben, wurde jeweils 1 Tropfen Patientenserum gebracht und 2 Stunden in der feuchten Kammer bei 37° C inkubiert. Anschließend Abspülen des Serums durch Eintauchen der Objektträger in gepufferte Kochsalzlösung, jeweils 15 Min. bei dreimaligem Wechsel der Waschflüssigkeit.

Zum *direkten Test* Zugabe eines Tropfens vorbereiteten F-AHG-Serums in die angezeichneten, feucht belassenen Areale des Objektträgers. Nach erneuter Inkubation über 60 Min. bei 37° C in der feuchten Kammer Abspülen des AHG-Serums durch intensive Waschung in Kochsalzlösung. Beurteilung der spezifischen Immunfluoreszenz der Leukocyten im Fluoreszenzmikroskop.

Beim *indirekten Test* wurde nach dem Auswaschen des Patientenserums das normale, also nicht fluoreszierende AHG-Serum vom Kaninchen dem Leukocytenpräparat zugesetzt, und zwar in einer Verdünnung von 1 : 10 in physiologischer Kochsalzlösung. Inkubation des Ansatzes 1 Stunde bei 37° C und nachfolgende, intensive Waschung in physiologischer Kochsalzlösung. Schließlich wurde ein Tropfen des Fluorescein-konjugierten Anti-kaninchen-Globulins vom Schaf zugegeben und nochmals 1 Stunde bei 37° C inkubiert. Die weitere Behandlung des Ansatzes erfolgte wie für den direkten Test beschrieben.

Die Spezifitätsprüfung positiver Befunde im Inhibitions- bzw. Adsorptionstest wurde in der für die histochemische Anwendung des F-AHG-T angegebenen Weise (16) durchgeführt.

Fluoreszenzmikroskopie. Die Untersuchung der fertigen Präparate geschah mit Hilfe des Mikroskopes »Ortholux« der Firma Leitz, Wetzlar, und dem dazugehörigen Fluoreszenzansatz. Als Lichtquelle diente die Quecksilberhochdrucklampe HB 200 (Osram) Blauglas-Erregerfilter BG 12 bei gelbem Sperrfilter. Mikroaufnahmen mit der Leitz-Photoeinrichtung Orthomat.

Weitere serologische Methoden

1. *Leukocyten-Agglutinations-Test* (LAT) unter Verwendung der kürzlich beschriebenen, eigenen Methodik (31). Inkubation der Seren mit den Spenderleukocyten 1 Stunde bei 37° C in silikonisierten Glasröhrchen. Bewertung der Testergebnisse an Hand der Stärke des Agglutinationsphänomens.

2. *Anti-Humanglobulin-Konsumptions-Test* (AGCT) (57) unter Verwendung der Technik von Moulinier (49). Antigen: lyophilisierte, hochgereinigte Leukocytenpräparation (41), die von Dr. Mathey, Blutspenderzentrum der französischen Armee, Paris, in dankenswerter Weise zur Verfügung gestellt wurde. Auf Grund der eigenen Erfahrung wurde ein positives Ergebnis registriert, wenn ein AHG-Serum-Verbrauch von mindestens 3 Dilutionsstufen eingetreten war. Bei 2 Dilutionsstufen Verbrauch galt der Test als schwach positiv, bei einer Stufe und weniger als negativ.

3. *Verbesserter-Latex-Test* als Tropfentest mit Patientenserum und Reagens der Fa. Hyland, Los Angeles.

Ergebnisse

Im Rahmen der Versuchsreihe konnten einige wichtige, technische Voraussetzungen erarbeitet werden, bei deren Berücksichtigung mit dem F-AHG-Test zufriedenstellend reproduzierbare Ergebnisse zu erzielen sind.

Die *Fixation* der Leukocytenausstriche mit unterkühltem Aceton erwies sich als optimale Methode, die bei ausreichendem Fixationseffekt ein Minimum an Fluoreszenz durch unspezifische Anlagerung der F-AHG-Seren an die Leukocyten mit sich brachte. Fixationsversuche mit Äthyl- bzw. Methylalkohol mußten wegen der teilweise stark ausgeprägten, unspezifischen Fluoreszenzeffekte, die sich auch durch nachhaltige Adsorptionsbehandlung der Antiseren – bei oft erheblichem Titerverlust – nicht beeinflussen ließen, wieder aufgegeben werden. Aceton-fixierte Leukocytenpräparationen sind offenbar nur beschränkte Zeit, auch bei niedrigen Temperaturen (—20° C), haltbar. Länger als 24 Stunden aufbewahrte Ausstriche ließen mit positiven Kontrollseren keine eindeutige Immunfluoreszenz mehr erkennen.

Durch Vorbehandlung der fluoreszierenden *Antiseren* mit Schweineleber-Organpulver und menschlichen Leukocyten war eine weitgehende, für die Differenzierung der

Unter den 25 *polytransfundierten* Patienten erbrachte der direkte F AHG-Test mit Vollserum vom Kaninchen 7mal ein deutlich positives Ergebnis. In 17 Fällen wurde ein schwach positiver Befund registriert, ein Serum war negativ Bei Anwendung des indirekten Verfahrens war der Anteil der stark positiven Ausfälle mit 13 Seren deutlich höher als im direkten Test. 10 Seren ergaben ein schwach positives Resultat, in 2 Fällen fiel der Test negativ aus.

Der spezifische Fluoreszenzeffekt ließ sich in allen positiven Fällen im Bereich des Leukocytenplasmas lokalisieren. Eine isolierte Kernfluoreszenz der Leukocyten konnte in keinem Fall beobachtet werden.

Mit Hilfe des LAT war in 10 Fällen dieser Gruppe ein sehr stark positiver Ausfall zu beobachten, 6mal fiel der Test deutlich, gleich oft schwach positiv aus. 3 Seren führten zu keiner Leukocytenagglutination.

Der AGCT verlief 4mal sicher positiv – Verbrauch von mehr als 2 Dilutionsstufen des AHG-Serums – 10mal schwach positiv und 5mal negativ (bei 6 Seren wurde der AGCT nicht durchgeführt) Die Ergebnisse der einzelnen Tests sowie die Korrelation der Befunde zur Frequenz der stattgehabten Transfusionen sind in Abb. 1 zusammengestellt.

Bei 8 Kranken mit klinisch gesichertem *Lupus erythematodes disseminatus* zeigte der F AHG-Test bei direktem und indirektem Ansatz in allen Fällen ein stark positives Ergebnis. Die spezifische Fluoreszenz der Leukocytenkerne als ein für den LED charakteristischer Befund, war in 3 Fällen besonders deutlich. Bei den restlichen Seren wurde, vor allem bei Anwendung des indirekten Testes, ein besonders intensiver Fluoreszenzeffekt der Gesamtzelle beobachtet, der möglicherweise die Fluoreszenz des Leukocytenkernes überlagerte.

Der LAT war in dieser Gruppe, von einer Ausnahme abgesehen, negativ Der AGCT erwies sich in 5 Fällen als positiv in einem weiteren als negativ (2 Seren konnten nicht untersucht werden). Der Thymonukleoprotein-Latex Test (Hyland) führte 6mal zu einem positiven Ergebnis, 2mal fiel er negativ aus.

Bei 5 weiteren Kranken bestand klinisch starker Verdacht auf das Vorliegen eines LED bzw einer Erkrankung aus dem Formenkreis der Kollagenosen, ohne daß die Diagnose serologisch und durch Nachweis des LE-Zell-Phänomens einwandfrei zu sichern war Zwei dieser Seren wiesen deutlich positive F AHG-Tests auf,

Abb. 1. Zusammenstellung der Ergebnisse mit dem Fluoreszenz-Anti-Humanglobulin-Test ( ), dem Leukocytenagglutinationstest ( ) und dem Anti-Humanglobulin-Konsumptionstest (+) bei 19 polytransfundierten Patienten in Korrelation zur Zahl der stattgehabten Bluttransfusionen. I der Vertikalen = 1 Patientenserum.

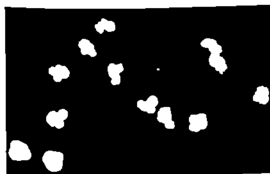


Abb. 2. Positive Kernfluoreszenz der Leukocyten im Fluoreszenz-AHG-Test bei einem Fall von Lupus erythematodes disseminatus.

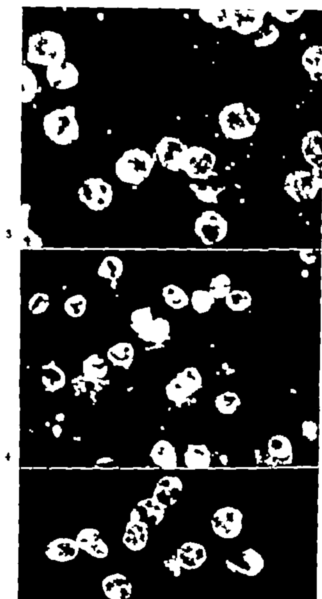


Abb. 3–5. Plasmafluoreszenz der Leukocyten im AHG-Test bei Agranulocytose (3), Transfusions-bedingten Isoantikörpern (4) bzw. Kälte-Agglutinin-Syndrom (5)

3 weitere waren schwach positiv. Dabei ließ sich vor allem in einem dieser Fälle eine intensive Kernfluoreszenz beobachten. Der ACGT ergab 2 positive und 3 schwach positive Resultate, einer dieser Verdachtsfälle hatte einen positiven LAT.

In 7 Fällen von *Agranulocytose* und bei 2 weiteren Kranken mit Verdacht auf medikamentös ausgelöste Immunleukopenie waren die Ergebnisse mit dem direkten F AHG Test uneinheitlich Ein Kranker dieser Gruppe bot eine deutlich positive Reaktion, ein weiterer bei dem klinisch Verdacht auf eine Immunleukopenie bei INH Behandlung bestand, ließ ebenfalls eine deutlich ausgeprägte Immunfluoreszenz der Testleukocyten erkennen 5 Seren lieferten ein nur schwach positives Ergebnis, 2 waren negativ Die Verwendung der indirekten Methode führte zu einem im wesentlichen identischen Ergebnis.

Der LAT fiel in 4 Fällen dieser Gruppe mit der Mehrzahl der getesteten Spenderleukocyten positiv aus Die restlichen Seren führten nur in Ausnahmefällen bei Verwendung von Leukocytensuspensionen einzelner Spender zu mehr oder weniger stark positiver Agglutination, so daß die Ergebnisse lediglich als bedingt positiv zu bewerten waren. Der AGCT brachte nur bei einem Serum ein deutlich positives, bei 3 weiteren Fällen ein sch ach positives Resultat. Bei den restlichen Seren ließ die Methode im Stich

Bei 5 Patienten des allgemeinen Krankengutes, die klinisch keinerlei Hinweise auf eine Immunopathie des Blutes, insbesondere des leukocytären Systems, erkennen ließen wurde ein positiver Ausfall des F AHG-Testes beobachtet. Unter diesen 2 Patienten boten zwei das typische Bild der plurionfiziellen Ektodermose (STEVENS-JOHNSON-Syndrom) 2 weitere litten an einem sogenannten Kälte-Agglutinin-Syndrom» Beim letzten Kranken dieser Gruppe bestand eine fortgeschrittene Dermatomyositis. In allen Fällen handelte es sich um teilweise ausgeprägte, spezifische Fluoreszenz des Leukocytenplasmas, die sich sowohl mit der direkten als auch mit der indirekten Methodik darstellen ließ Leukocyten-Agglutinine konnten in allen 5 Fällen nachgewiesen werden Bei den Kranken mit Kälte Agglutinin-Syndrom fiel der LAT bei Temperaturen zwischen 20 und 4 C besonders stark positiv aus.

Diskussion

Ein dem klassischen Anti Humanglobulin-(AHG) Test («COOMBS-Test») analoges Verfahren zum Nachweis leukocytenspezifischer Antikörper hat sich bisher technisch nicht realisieren lassen. Stattdessen nimmt der AHG-Konsumptions-Test (AGCT) in seinen verschiedenen, technischen Modifikationen (20 39 49

57) in der serologischen Praxis einen breiten Raum ein, während andere Verfahren leukocytäre Iso- bzw. Auto-Antikörper nachzuweisen, demgegenüber stark in den Hintergrund treten (45). So hat die Methodik, mit Hilfe Fluoreszein-markierter AHG-Seren spezifisches Antikörper-γ-Globulin an Leukocyten fluoreszenzoptisch cytochemisch darzustellen, bis heute kaum praktische Bedeutung erlangt. Dabei bietet die Fluoreszenz-serologische Technik gegenüber zahlreichen anderen Nachweismethoden auf diesem Gebiet wesentliche Vorteile, die eine umfangreichere Anwendung als bisher gerechtfertigt erscheinen lassen.

Allerdings sind, wie die vorliegenden Untersuchungen ergeben haben, einige wichtige Gesichtspunkte hinsichtlich der technischen Durchführung des Testes zu berücksichtigen, damit ein einwandfreies Funktionieren gewährleistet ist. Die Testleukocyten müssen vor ihrer Verwendung sorgfältig in einer geeigneten Salzlösung eiweißfrei gewaschen werden, da Plasmaproteinreste an der Leukocytenoberfläche (2, 52, 59) unter Umständen zu falsch positiven Reaktionen mit AHG-Seren führen können, wie in früheren Untersuchungen gezeigt wurde (30). Dabei muß der Waschprozeß so schonend wie möglich vor sich gehen, damit die Integrität der Zellen gewahrt bleibt. Die kürzlich angegebene Methode zur Isolierung menschlicher Leukocyten aus peripherem Blut (31) wird diesen Forderungen weitgehend gerecht. Zur Fixation der Leukocytenausstriche ist unterkühltes, wasserfreies Aceton anderen Fixationsmitteln vorzuziehen, da bei diesem Vorgehen die Tendenz der Leukocyten, fluoreszierende Antiseren unspezifisch anzulagern (42) auf ein Minimum beschränkt bleibt. Durch Adsorptionsbehandlung der Antiseren mit Organpulver aus Schweineleber bzw. menschlichen Leukocyten ist die unspezifische Fluorochromierung der Testzellen auf ein Mindestmaß zu reduzieren, so daß sie bei der Beurteilung der Immunfluoreszenz nicht mehr stört. Dabei hält sich der mit der Vorbehandlung verbundene Titerverlust der Antiseren in annehmbaren Grenzen.

Die Prüfung des F-AHG-Testes im Rahmen der immunhämatologischen Diagnostik an einem vorerst zahlenmäßig noch beschränkten Untersuchungsgut hat seine Brauchbarkeit unter Beweis gestellt. Bei der Untersuchung von Seren polytransfundierter Kranker sowie bei Fällen von Agranulocytose bzw. Immunleukopenie konnte in einem vergleichsweise hohen Prozentsatz der Antikörpernachweis fluoreszenzserologisch erbracht werden. Aller

dings fiel der Anteil schwachpositiver Reaktionen, vor allem bei Anwendung der direkten Technik, zum Teil noch relativ hoch aus. Die Zuverlässigkeit des F AHG-Tests beim Nachweis von LED-typischen Auto-Antikörpern ist mehrfach beschrieben worden (1 4 11 12, 23 26 55 59 60) In den eigenen Versuchen mit Leukocyten als Testsubstrat konnte die hohe Empfindlichkeit bestätigt werden. Außerdem fiel die besonders intensiv ausgeprägte Immunfluoreszenz an den Testleukocyten beim Arbeiten mit diesen Seren auf.

Die spezifische «apfelgrüne» Immunfluoreszenz war in den meisten positiven Fällen – so beim Nachweis transfusionsbedingter Iso-Antikörper (s. Abb. 4) sowie bei den untersuchten Agranulocytose-Seren (s. Abb. 3) – im Bereich des Leukocytenplasmas zu beobachten, ein Befund, auf den bereits früher wiederholt hingewiesen wurde (1 11 12, 24 50) Bei 3 LED-Seren kam es dagegen zu einer mehr oder weniger selektiven, spezifischen Fluorochromierung der Leukocytenkerne (s. Abb. 2) während bei den restlichen Fällen von LED lediglich eine, meist sehr intensive, spezifische Fluoreszenz der Gesamtzellen auftrat, ohne daß sich der Zellkern fluoreszenzoptisch näher differenzieren ließ. Es liegt nahe, diesen Befund, dessen nähere Analyse durch Adsorptionsversuche und Anwendung des F AHG-Tests auf einzelne Zellfraktionen noch aussteht (24 51) auf leukocytäre Antikörpergemische, deren Spezifität gegen verschiedene Zellbestandteile des Leukocyten gerichtet ist, zurückzuführen, wobei technisch-präparative Ursachen ebenfalls in Erwägung zu ziehen sind.

Bei optimaler technischer Ausführung des F AHG-Tests war der Anteil falsch-positiver Ergebnisse im Rahmen der Untersuchungen an Kontrollseren relativ gering Dabei scheint das indirekte Verfahren etwas mehr belastet zu sein als das direkte Welche Ursachen den unspezifisch-positiven Reaktionsausfällen im einzelnen zugrunde liegen und ob Beziehungen zu bestimmten Erkrankungsformen, z. B. Leberaffektionen, Dysproteinämien usw bestehen, bleibt anhand eines größeren Untersuchungsgutes abzuklären.

Auf Grund der bisherigen Erfahrungen mit der Fluoreszenz serologischen Methodik kann festgestellt werden, daß der F AHG-Test ein hohes Maß an Spezifität besitzt. Bei der teilweise doch ausgeprägten Tendenz einiger Leukocyten-serologischer Untersuchungsmethoden zu unspezifisch positiven Reaktionen (59) ist diese Tatsache besonders hervorzuheben. Die Empfindlichkeit des Tests

ist zwar je nach der Art der nachzuweisenden Antikörper unterschiedlich im ganzen gesehen jedoch für praktisch-diagnostische Zwecke ausreichend. Im Hinblick auf die hohe Sensibilität beim Nachweis LED-typischer Antikörper erscheint er als Suchreaktion bei klinischen Verdachtsfällen besonders geeignet. Auf Grund seiner serodiagnostischen Breite kann man mit seiner Hilfe praktisch das gesamte Spektrum leukocytenspezifischer Antikörper erfassen, was deshalb von Wichtigkeit ist da zahlreiche andere Methoden zum Nachweis des LED erfahrungsgemäß infolge ihrer hohen Spezifität relativ wenig sensibel sind oder vor allem in der akuten Phase der Erkrankung, durch Hemmung der leukocytären Phagocytose Vorgänge negativ bleiben können.

Positive serologische Befunde bei der Agranulocytose bzw der medikamentösen Immunleukopenie werden im allgemeinen selten erhoben. Nach der Erfahrung der meisten Autoren läßt der Leukocyten Agglutinationstest vielfach im Stich, auch der Anti Humanglobulin Konsumptionstest bringt nur in einem geringen Prozentsatz der Fälle positive Resultate (18, 35 39 45 53 59) Die vorliegenden Untersuchungsergebnisse berechtigen zu der Annahme daß gerade für die Agranulocytose und verwandte Krankheitszustände von der Einbeziehung des F AHG Tests in die serologischen Routinemethoden unter Umständen ein diagnostischer Fortschritt zu erwarten ist.

In 2 Fällen von Kälte Agglutinin-Syndrom konnte das gegen die Leukocyten gerichtete Prinzip durch positiven F AHG Test und spezifische Fluoreszenz des Zellplasmas nachgewiesen werden (Abb. 5) Früher mehrfach geäußerte Zweifel an der Existenz Leukocyten-aktiver Kryoagglutinine, die vor allem auf Grund der Kritik an der Methode des LA Tests («mixed agglutination») (10) laut wurden, konnten durch die Fluoreszenz serologischen Befunde ausgeräumt werden (32) Den wenigen im Schrifttum veröffentlichten Fällen von Dermatomyositis (12 26) mit Fluoreszenz serologischem Antikörpernachweis gegen Kernsubstanzen konnte ein eigener hinzugefügt werden, dessen Serum einen positiven F AHG-Test mit Leukocyten aufwies. Dabei muß offenbleiben, ob es sich in diesen Fällen um Antikörper identischer Spezifität handelt. Durch positive Reaktion im F AHG Test fielen weiterhin 2 Patienten auf, die klinisch das Vollbild des sogenannten «Stevens-Johnson-Syndroms» boten. Positive serologische Befunde an Leukocyten bei dieser Erkrankung sind unseres Wissens bisher nicht beschrieben worden.

Die Annahme liegt nahe daß es sich hier um den Ausdruck einer partiellen Gemeinschaft von Haut und Leukocyten-Antigenen handelt, die von der Transplantationsimmunologie her bekannt ist (21 59) Die weitere Klärung dieser Frage dürfte für die Kenntnis der Pathogenese des Krankheitsbildes von Interesse sein.

Die Gegenüberstellung der Untersuchungsergebnisse mit dem LA Test, dem AGC-Test und dem F AHG-Test gestattet aus methodischen Gründen lediglich einen orientierenden Überblick und erlaubt keine detaillierten Rückschlüsse hinsichtlich der Aussagefähigkeit der einzelnen Methoden für die immunhämatologische Praxis. Auffällig ist aber der relativ hohe Anteil positiver Resultate des LA Tests in der Gruppe der polytransfundierten Kranken, was die Empfindlichkeit der Methode beim Nachweis transfusionsbedingter Isoantikörper erneut unter Beweis stellt (18 35 53 59) Andererseits konnte an Hand der Versuche die hinreichend bekannte, oft viel zu wenig in Rechnung gestellte Tendenz des LA Tests zu unspezifisch-positiven Ausfällen aufs neue bestätigt werden (8 33 35 59) Der wesentliche Vorteil der Fluoreszenz immunologischen Methodik für die Serologie der Leukocyten liegt in der Möglichkeit, nicht nur komplette (agglutinierende) sondern auch inkomplette (univalente) Antikörper zu erfassen wie sie als Auto-Antikörper bei den verschiedensten Krankheitszuständen vorkommen. Darüberhinaus wird man in Zukunft mit Hilfe des Tests Antikörper gegen einzelne Zellelemente der leukocytären Reihe unterschiedlicher Herkunft (und verschiedenen Reifegrades?) fluoreszenzoptisch differenzieren, sowie leukocytäre Antikörper die gegen Partialantigene der Leukocytenzelle gerichtet sind, nachweisen und lokalisieren können. Bis sich diese, auf einigen Arbeitsgebieten (5 7 15 28, 36 43 44 58) bereits mit Erfolg wahrgenommenen methodischen Vorzüge in vollem Umfange ausschöpfen lassen, werden noch zahlreiche Verbesserungen der präparativen Voraussetzungen sowie eine weitere Anpassung der Fluoreszenz serologischen Technik an die Erfordernisse immunhämatologischer Arbeitsmethoden erforderlich sein

Zusammenfassung

Es werden technische Richtlinien zur Durchführung eines Fluoreszenz-serologischen Anti-Humanglobulin-Tests an menschlichen Leukocyten angegeben und erste Erfahrungen mit diesem Test an einem immunhämatologischen Untersuchungsgut mitgeteilt. Die Vorteile des Tests für die Serodiagnostik von Immunopathien der Leukocyten werden diskutiert.

Summary

Technical directions for carrying out fluorescent antiglobulin test on human leukocytes are given together with report on experience with this test in an immunological investigation. The advantages of this method for serological diagnosis of leukocyte immunopathy are discussed.

Résumé

Les indications techniques pour un test antiglobulinique fluorescent avec des globules blancs humains, ainsi que les premiers résultats d'expériences immunologiques avec ce test sont communiqués. On discute les avantages de cette méthode pour le sérodiagnostic de l'immunopathie leucocytaire.

Literatur

1. Alexander, W. R. M., Bremner, J. M. and Duthie, J. J. R. Incidence of the anti-nuclear factor in human sera. Ann. rheum. Dis. *19*: 338–350 (1960).
2. Anderson, R. E. and Walford, R. L. Detection of leukocyte antibodies by means of I^{131} labelled purified anti-human-globulin antibody. Problem of nonspecific adsorption of globulin by leukocytes. Blood *16*: 1523–1534 (1960).
3. Anderson, R. E., Walford, R. L. and Doyle, P. C. Leukocyte antibody in acute leukemias of monocytes and monocytoid forms. Amer. J. clin. Path. *36*: 25–30 (1961).
4. Barnes, R. and Carmichael, D. Comparison between the latex nucleoprotein test and the fluorescent method for the demonstration of antinuclear factor. Ann. rheum. Dis. *21*: 287–291 (1962).
5. Barnhart, M. I. and Riddle, J. M. Cellular localization of profibrinolysin. Blood *21*: 306–321 (1963).
6. Bock, H. E. Agranulocytose. Vorträge aus der praktischen Medizin. F. Enke, Stuttgart (1946).
7. Braunsteiner, H., Riegler, R. und Pakesch, F. Immunfluoreszenz-Untersuchungen an Knochenmarksausstrichen bei primär chronischer Polyarthritis. Klin. Wschr. *40*: 1139–1141 (1962).
8. Brittingham, T. E. Observations on mechanism and prevention of non specific agglutination of leukocytes. Proc. Soc. exp. Biol., N. Y. *99*: 252–254 (1958).
9. Brittingham, T. E. and Chaplin, H. Antigenicity of normal and leukemic human leukocytes. Blood *17*: 139–165 (1961).
10. Butler, J. J. Some studies on the naturally occurring leukocyte agglutinins. J. clin. Invest. *35*: 1150–1160 (1956).
11. Calabresi, P., Edwards, E. A. and Schilling, R. F. Fluorescent antiglobulin studies in leukopenic and multiple transfusion disorders. J. Lab. clin. Med. *52*: 801 (1958).
12. Calabresi, P., Edwards, E. A. and Schilling, R. F. Fluorescent antiglobulin studies in leukopenic and related disorders. J. clin. Invest. *38*: 2091–2100 (1959).
13. Calabresi, P. and Finch, S. C. The value of the fluorescent antiglobulin technic in the differential diagnosis of aleukemic leukemia. Proc. 8. Congr. int. Soc. Blood Transfus., Tokyo, pp. 324–329 (S. Karger, Basel 1962).
14. Chudomel, V., Jezkova, Z. and Libansky, J. Detection of leukocyte antibodies by the complement consumption test. Blood *14*: 672–690 (1959).
15. Cohen, F., Zuelzer, W. W. and Evans, M. M. Identification of blood group antigens and minor cell populations by the fluorescent antibody method. Blood *15*: 884–900 (1960).

16. Coons, A. H.: Antibodies and antigens labelled with fluorescein. Schweiz. Z. Path. Bakt. 22: 693–699 (1959). Fluorescent antibody methods; in Danielli' General Cytochemical Methods, pp. 400–424 (Academic Press, New York 1958).
17. Coons, A. H. and Kaplan, M. H.: Localization of antigen in tissue cells. J. exp. Med. 91: 1–13 (1950).
18. Dausset, J.: Immuno-hématologie, biologique et clinique (Flammarion, Paris 1956).
19. Dausset, J.: Iso-leuco-anticorps. Acta haemat. 20: 156–166 (1958).
20. Dausset, J. and Colombani, J. and M.: Study of leukopenias and thrombocytopenias by the direct antiglobulin consumption test on leukocytes or/and platelets. Blood 17: 672–690 (1961).
21. Dausset, J. et Colombani, J.: Isoanticorps antileucocytaires et antiplaquettaires. Sérologie et importance pratique en particulier pour les greffes. Proc. 8. Congr. int. Soc. Blood Transfus., Tokyo, pp. 324–329 (S. Karger, Basel 1962).
22. Dausset, J.; Nenna, A. et Brecy, H.: Leuko-agglutinins. Blood 9: 696–720 (1954).
23. Doom, C.; Engelfriet, P. and van Loghem, J. J.: Immunological procedures for the demonstration of the LE-factor and leukocyte antibody. Ned. T. Geneesk. 105: 1779–1786 (1961).
24. Finch, S. C. and Dexter, E. D.: The localization and action of leukocyte antibody. Vox Sang. 13: 42–47 (1958).
25. Fleck, L.: Über Leukergie. Acta haemat. 8: 282–293 (1952).
26. Friou, G. J.: The significance of the lupus-globulin, nucleoprotein reaction. Ann. intern. Med. 49: 866–875 (1958).
27. Goldstein, G.; Spalding, B. H. and Hunt, W. B.: Studies on fluorescent antibody staining. Proc. Soc. exp. Biol., N. Y. 111: 416–421 (1962).
28. Greenspan, J. and Schwartz, S. O.: Immunologically specific antigens in leukemic tissues. Blood 21: 717–728 (1963).
29. Gross, R. und Bock, H. E.: Erkrankungen der Leukopoese und des reticulo-histiocytären Systems. Klin. Gegenw. 10: 567–643 (1962).
30. Hartl, W.: Zur Frage eines direkten Anti-Humanglobulin-Testes an isolierten menschlichen Leukocyten. Acta haemat. 27: 104–112 (1962).
31. Hartl, W.: Zur Technik des Leukocyten-Agglutinationstestes. Acta haemat. 28: 273–282 (1962).
32. Hartl, W.: Serologische Untersuchungen an Leukocyten bei einem Fall von Kälteagglutinin-Syndrom. Klin. Wschr. 41: 801–805 (1963).
33. Hartman, J. D.: An in vitro technic for quantitating and studying the dynamics of leukocyte-agglutination. Blood 13: 447–456 (1958).
34. Holborow, E. J.; Weir, D. M. and Johnson, G. D.: A serum factor in lupus erythematosus with affinity for tissue nuclei. Brit. med. J. ii: 732 (1957).
35. Killmann, S.: Leukocyte Agglutinins (Blackwell, Oxford 1959).
36. Kuroyanagi, T.; Kurisu, A. and Sugiyama, H.: Evidence for the in vivo localization of heterologous antileukocyte and anti-bone marrow antibodies in the rat bone marrow. Blood 21: 594–604 (1963).
37. Jankovic, B. D. and Arsenijevic, K.: The demonstration of antileukocyte antibodies in human and rabbit immune serums by means of fluorescent antibody technique. Acta haemat. 21: 387–393 (1959).
38. Lee, S. L.: Inhibition of leukocyte agglutination by serum from patients with systemic lupus erythematosus. A manifestation of the LE-cell phenomenon. Blood 11: 778–783 (1956).
39. van Loghem, J. J.; van der Hart, M.; Hijmans, W. and Schuit, H. R. E.: The incidence and significance of complete and incomplete white cell antibodies with special reference to the use of the Coombs consumption test. Vox Sang. 3: 203–211 (1958).

40. Marshall, J. D., Eveland, W. C. and Smith, Ch. W.: Superiority of fluorescein-isothiocyanate (Riggs) for fluorescent antibody technic with a modification of its application. Proc. Soc. exp. Biol., N. Y. 98, 898–900 (1958).
41. Maupin, B.: Au sujet de la séparation des leucocytes à partir du sang humain. Sang 26, 328–336 (1955).
42. Mayersbach, H.: Unspecific interactions between serum and tissue sections in the fluorescent antibody technic for tracing antigens in tissues. J. Histochem. Cytochem. 7, 427 (1959) cit. Blood 16, 1238 (1960).
43. Mellors, R. C.; Heimer, R.; Corcos, J. and Korngold, L.: Cellular origin of rheumatoid factor. J. exp. Med. 110, 875–886 (1959).
44. Mellors, R. C.; Nowoslawski, A.; Korngold, L. and Sengson, B. L.: Rheumatoid factor and the pathogenesis of rheumatoid arthritis. J. exp. Med. 113, 475–483 (1961).
45. Miescher, P. und Vorlaender, K. O.: Immunopathologie in Klinik und Forschung (Thieme, Stuttgart 1961).
46. Mizarcos, F.; Palester, M.; Wazniewska, G. and Dudziak, Z.: Complement fixing leukocyte antibodies. Vox Sang. 2, 263–267 (1957).
47. Moeschlin, S.: Immunoleucopénies et immunogranulocytoses. Rev. Hémat. 8, 249–262 (1953).
48. Moeschlin, S.: Leukocyte-autoantibodies. Acta haemat. 20, 167–178 (1958).
49. Moullinier, J.: Technique de la réaction de consommation d'antiglobuline. Rev. franç. Et. clin. biol. 2, 355–364 (1956).
50. Müller, W. und Schirrmacher, H.: Eine einfache Methode zur Durchführung des Antiglobulintests an Leukocyten. Klin. Wschr. 38, 403–404 (1960).
51. Nathan, D. J. and Shapiro, J.: On the interaction of dead leukocyte nuclei, LE-factor and living leukocytes in the LE-cell phenomenon. Blood 13, 883–893 (1958).
52. Nelken, D.: The nature of the coating globulin causing a positive antiglobulin consumption test. J. med. Ass. Israels 60, 83–84 (1961) cit. Blood 18, 131 (1961).
53. Payne, R.: Leukocyte agglutinins in human sera. Arch. intern. Med. 99, 587–606 (1957).
54. Riggs, Kirchhofer, H.: Ultra-rapid fluorescent labelling of proteins. Nature 193, 167–168 (1962).
55. Seligmann, M.: Anticorps anti-leukocytaires du lupus erythémateux disséminé. Acta haemat. 24, 81–92 (1960).
56. Smith, Ch. W.; Marshall, J. D. and Eveland, W. C.: Use of contrasting fluorescent as counterstain in fixed tissue preparations. Proc. Soc. exp. Biol., N. Y. 102, 179–181 (1959).
57. Steffen, C. und Schindler, H.: Bericht über die Verwendung des Antihumanglobulin-Ablenkungsversuches für den Nachweis eines Antileukocytenantikörpers bei Agranulocytosen. Münch. med. Wschr. 97, 469–471 (1955).
58. Vasquez, J.: Immunocytochemical study of plasma cells in multiple myeloma. J. Lab. clin. Med. 51, 271–275 (1958).
59. Walford, R. L.: Leukocyte antigens and antibodies (Grune and Stratton, New York/London 1960).
60. Weir, D. M.; Holborow, E. J. and Johnson, G. D.: A clinical study of serum antinuclear factor. Brit. med. J. 5230, 933–937 (1961).

Adresse des Autors: Dr. W. Hartl, Medizinische Klinik der Universität, Otfried-Müller-Strasse, 74 Tübingen (Deutschland).

Acta haemat. 30 305–309 (1963).

From Clinic I, Vasa Hospital and Central Chemical Laboratory and Blood Bank,
Sahlgrens Hospital, Gothenburg

Oxidation of Haemoglobin by Hydralazine plus Leukocytes and its Facilitation in Seras from Hydralazine Sensitive Patients

By P. Nordqvist, E. Persson, L. Ryttinger and M. Ljunggren

Sodium ethylenediaminoacetic acid (EDTA) and leukocytes added to a suspension of erythrocytes or a solution of oxyhaemoglobin oxidize the haemoglobin to methaemoglobin (2). The scope of the present investigation is to show that another metal binding agent hydralazine, widely used in medical therapy, can substitute for EDTA in this process. Further will be shown that presence of serum from patients sensitive to hydralazine facilitates this reaction.

Material and Methods

1. The experimental arrangement and the solutions used have previously been described in detail (2).

2. *Hydralazine solution:* 50 mg Apresoline® was dissolved in 10 ml. saline. Approximately 0.05 ml of this solution was added to 10 ml erythrocyte suspension or haemoglobin solution.

3. *Sera from cases hypersensitive to hydralazine.* Two patients were admitted to the hospital with haemolytic anaemia, where the medical history and course strongly indicated the anaemia to be due to hydralazine sensitivity. The sera were obtained from these patients since the effect of hydralazine on the haemoglobin was noticed by chance when serological investigations were carried out. T serum from case 1 (S.K. 21 06 21 / 61 Med. clin. I), erythrocytes and leukocytes from donors and hydralazine were added. The serum of case 2 (H.H. 11 07 03 / 60 Med. clin. I) was at the date of the tests haemolytic to such an extent that it could be used directly as an oxyhaemoglobin solution. Only limited amounts of samples were available.

4. *Leukocyte and erythrocyte suspensions and control sera:* With one exception reported below, they were prepared from healthy donors. The leukocyte suspensions according to method described by Nordqvist et al. 1961 (3) and the erythrocyte suspensions and control sera as described by Nordqvist et al. 1963 (2).

Results

Hydralazine participates in changing oxyhaemoglobin into methaemoglobin when added with leukocytes to an erythrocyte

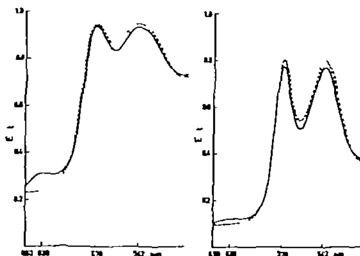


Fig 1 Left: Mixture of oxyhaemoglobin solution, serum and leukocytes from donor with hydralazine. *Right:* Mixture of erythrocytes, serum and leukocytes from donor with hydralazine. Incubation time 9 hours at 37 C. Sraight line indicates before, dotted line after addition of cyanide.

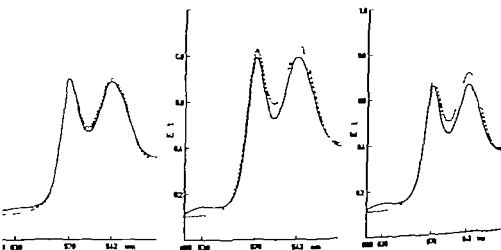


Fig 2. Left: Mixture of erythrocytes from case 1 serum and leukocytes from donor and hydralazine. *Middle:* Mixture of oxyhaemoglobin solution, serum and leukocytes from donor with hydralazine. *Right:* Mixture of erythrocytes and leukocytes from donor, serum from case 1 and hydralazine.
Stright line indicates before, dotted line after addition of cyanide.

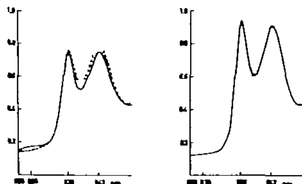


Fig. 3. Left: Mixture of serum from case 2 with leukocytes from donor and hydralazine. *Right:* Mixture of serum from case 2 and leukocytes from donor. Incubation time 3 hours at 37 C. Straight line indicates before, dotted line after addition of cyanide.

suspension or an oxyhaemoglobin solution. This reaction is far more rapid, when the oxyhaemoglobin is free in solution than when it is bound inside the erythrocytes (fig 1)

However when the reaction was carried out on intact erythrocytes suspended in serum from case 1 the speed of the reaction was almost as rapid as when an oxyhaemoglobin solution was used. Contrary to this any facilitation of the reaction was not observed when erythrocytes from case 1 were immersed in serum from a healthy donor (fig 2)

Hydralazine added with leukocytes directly to serum from case 2 oxidated the ferrohaemoglobin to ferrihaemoglobin, which did not happen when only leukocytes were added (fig 3)

Discussion

Hydralazine is a chelating agent (1) containing aminogroups like EDTA. A possible explanation for the action of these substances in the oxidation of ferrohaemoglobin to ferrihaemoglobin has been given previously (2) At the time the seras of the patients were investigated they contained no methaemoglobin before addition of hydralazine and leukocytes. Therefore we do not know if formation of methaemoglobin had any significance for the toxic reaction, in these cases, or not. However a large number of useful drugs possess

Acta haemat. 30: 310–316 (1963).

Laboratory for Blood Morphology and Cytology The Kaplan Hospital of the Labourer's Sick Fund, Rehovoth

Acid Phosphatase Activity in Normal Human Blood and Bone Marrow Cells as Demonstrated by the Azo Dye Method

By L. ROZENSZAJN G MARSHAK AND P EFRATI

Acid phosphatase activity in peripheral blood and bone marrow cells, was first demonstrated by RABINOVICH et al. (1) and STORTI et al. (2) Both workers have used the method of GOMORI for this purpose, and have demonstrated enzyme activity only in bone marrow smears, while getting negative results in peripheral blood. HAIGHT (3) and VALENTINE et al. (4) using biochemical technics, have demonstrated enzyme activity in lymphocytes and granulocytes from peripheral blood. In 1962 LÖFFLER AND BERGHOFF (5) reported on use of Azo Dye for demonstration of acid phosphatase activity in cells of the haemopoietic system.

In the present study we tried to examine and estimate, both qualitatively and quantitatively acid phosphatase activity in peripheral blood and bone marrow cells. With the coupling Azo dye method of PEARSE (6) with several modifications, we showed enzyme activity in all the nucleated cells of the haemopoietic system.

Materials and Methods

Peripheral blood smears were taken from 25 healthy volunteers (nurses, medical students and hospital personnel) varying in age from 19—34 years, half of them males. Bone marrow smears were obtained from iliac crests of 18 patients, and all of them were within normal morphological limits, as demonstrated by the May-Grünwald-Giemsa staining technique. In addition, peripheral blood smears from 10 rats, and 10 C_3H mice were examined.

The substrate consisted of

Sodium alpha naphtyl acid phosphate 50 mg*
Fast-Garnet salt G.B.C. 50 mg*
Acetate buffer 0.1 M, pH 5 50 ml

The Borden Chemical Company USA.
George T Gurr Ltd., England

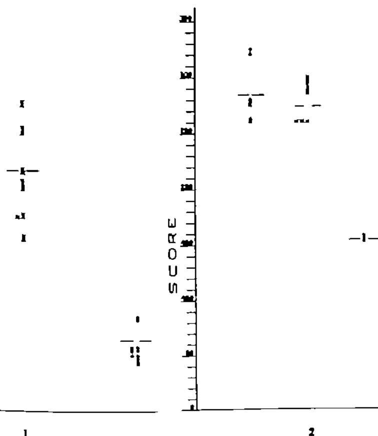


Fig. 1 Acid phosphatase activity in lymphocytes (o) and neutrophil granulocytes ( ) in peripheral blood.

Fig. 2. Acid phosphatase activity in neutrophil granulocytes with around nucleus (), mature neutrophil granulocytes (×), pronormoblasts (•) and normoblasts in bone marrow

It was mixed thoroughly and after passing through filter paper was ready for use

All smears were allowed to dry in room air then fixed for 7 minutes in 40 formaldehyde vapour immediately washed in running tap-water for 3–5 minutes and allowed to dry Subsequently the smears were incubated in the substrate for 1 hour at 37° C, then were washed with tap-water and dried in air Contrast staining was done for 5 minutes with haematoxylin Harris, then the preparations were washed in tap-water and after being dried in air they were protected by cover glass with glycerine jelly

Acid phosphatase activity was demonstrated only in the cytoplasm of all cells (except erythrocytes) in the shape of brown granules of various size and occasionally in the form of dark brown crystals. When smears were treated with substrate without sodium alpha-naphthyl-phosphate, shining yellow pigment precipitates were seen in the cytoplasm, nucleus and intercellular space. This yellow pigment appeared entirely different from the granules expressing acid phosphatase activity

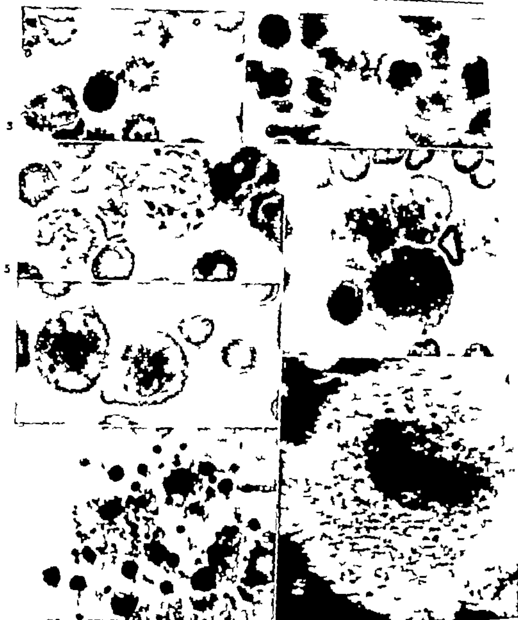


Fig 3 Lymphocyte with one central granule in the cytoplasma (peripheral blood).
Fig 4 Two neutrophil granulocytes in the center and at the margin monocyte and lymphocy (peripheral blood)
Fig 5 Myelocytes and metamyelocytes with strong acid phosphatase activity (bone marrow)
Fig 6 Young myeloid cells, strong acid phosphatase activity in eosinophilic myelocyte (bone marrow)
Fig 7 Pronormoblasts with slight acid phosphatase activity (bone marrow).
Fig 8 A mature megakaryocyt with strong acid phosphatase activity (bone marrow).
Fig 9 A reticulum cell with very strong acid phosphatase activity

Fixation of the smears for 5 minutes with methyl alcool destroyed all activity. Incubation at 4 °C showed low grade activity which increased when the temperature as raised to 20 °C and reached its maximum at 37 °C. At 56 °C, enzyme activity decreased to level similar to that seen at 4 C.

I all peripheral blood smears, 100 neutrophils and 100 lymphocytes were counted. In bone marrow smears in addition to 100 mature neutrophils, 100 granulocytes with *round nucleus* and 100 pronormoblasts were counted.

Acid phosphatase activity in each cell was graded as follows

0 no stained granules
\+ 1–2 intermediate granules or 1 5 small granules
++ 2–4 large granules or 4–8 intermediate granules
+++ 4–8 large granules or 8–16 intermediate granules or many small granules.
++++ More than that.

The sum of the score of 100 granulocytes or 100 lymphocytes or the other cells described above constitutes the score as used in this study

Results

Peripheral blood

1) Neutrophilic granulocytes revealed intermediate activity scattered in the cytoplasm in the form of brown granules, mostly small to intermediate in size but occasionally large there was no special arrangement of these granules, but occasionally they formed a frame surrounding the nucleus (fig 1 4)

2) Eosinophile granulocytes showed strong activity The eosinophilic «specific» granules stained greenish-grey and on that background were seen the dark brown granules, mostly large to intermediate in size and a lesser amount of smaller granules.

3) Lymphocytes presented slight activity a few small granules were observed (fig 1 3)

4) Monocytes disclosed strong activity the cytoplasm was full of granules (fig 4)

5) Thrombocytes acid phosphates activity was demonstrated as dark brown dots.

In the peripheral blood smears in 10 rats there was activity of 1–2 + in the mononuclear cells. The granulocytes showed no activity In the C_3H mice, both mononuclear cells and granulocytes demonstrate enzyme activity

Bone marrow

1) Young granulocytes Due to difficulties in estimating the stage of maturity the younger cells were considered together as «round nuclear» cells, these cells showed strong activity (fig 2, 5 6)

2) Mature granulocytes revealed strong activity (fig 2)

3) Pronormoblasts and basophilic normoblasts showed slight activity in the form of small granules (fig 2 7)

4) Mature normoblasts A small number of cells showed positive staining with one or several granules (fig 2)

Table I

		Score	Range of score	Degree of activity, 0	+	++	+++	++++
	Round nucleus cells	284	231 324	0.1	4.7	30.3	42.6	22.6
Bone	Mature neutrophils	273	217–300	1 1	7 7	30.4	37.8	22.9
Marrow	Pronormoblasts and basophilic normoblasts	147	92 188	10.7	40.7	39.2	8.3	0.7
cells	Mature normoblasts	32	4–56	70.1	28	1.9	0	0
Peripheral	Lymphocytes	65	21–91	40.7	54.2	4.6	0.4	0.1
Blood cells	neutrophilic granulocytes	217 7	140–305	0.8	28.7	34.3	29.6	6.6

5) Megakaryocytes Very strong acid phosphatase activity was demonstrated in the cytoplasm in the form of large, intermediate and small granules increasing with cellular maturity (fig 8)

6) Plasma cells Strong activity was demonstrated especially in the chromophobic zone.

7) Reticulum cells Very strong activity was demonstrated throughout the cytoplasm. In addition to granules, larger amorphous masses of brown material were seen. The latter on some occasions obscured the nucleus (fig 9)

8) Tissue mast cells showed very strong activity sometimes large masses of brown material obscured the nucleus.

The average degree of activity the score and the range of scores of peripheral blood and bone marrow is summarized in table I

Acid phosphatase activity in peripheral blood is summarized in fig 1 Acid phosphatase activity in bone marrow is summarized in fig 2.

Discussion

Acid phosphatase activity in peripheral blood was examined by HAIGHT et al. (3) using biochemical techniques with disodium phenyl phosphate I as substrate and by doing differential cell counts they were able to demonstrate that acid *phosphatase activity was greater* in lymphocytes than in granulocytes. Using the method described above we have found greater enzymatic activity in granulocytes as compared with lymphocytes (fig 2) The examination of acid phosphatase activity by STORTI (2) and RABINOVICH (1) in bone marrow and peripheral blood revealed activity in bone marrow only One may assume that our method is more sensitive, making it possible to demonstrate activity in the form of granules, rather than as a different staining as in the method of GOMORI (7) which they used. According to fig 2 one gets the impression that the enzyme activity is strongest in young cells and decreases with cell maturity both in the myeloic and erythropoietic cell lines, although the numerical differences between them in the myeloic cells are rather small. LÖFFLER AND BERGHOFF (5) who used fixation by cold aceton came to similar results.

On the other hand in the thrombopoietic system the enzymatic activity increases with cell maturity The percentage of mature granulocytes giving a 3 to 4 + positive reaction in the bone marrow is greater than the number of cells of the same type exhibiting a similar reaction in peripheral blood. One may speculate that this difference is due to difference in age

Summary

Acid phosphatase activity was demonstrated in all nucleated cells of human blood and bone marrow using an azo dye technique. The activity was greater in granulocytes than in lymphocytes. In the bone marrow young cells show greater enzyme activity than mature cells except for megakaryocytes where the reverse is true. The results obtained differ from those reported by others, presumably because of the greater sensitivity of the method employed. In addition, blood smears of rats and mice were examined.

Résumé

A l'aide d'un procédé au colorant azoïque on démontra la phosphatase acide dans toutes les cellules humaines nucléées du sang et de la moelle osseuse. L'activité de la phosphatase était plus intense dans les granulocytes. Dans la moelle osseuse l'activité enzymatique des cellules jeunes est plus grande que dans les cellules matures, sauf pour les mégacaryocytes chez lesquels on trouve le contraire. Les résultats obtenus diffèrent de ceux rapportés par d'autres auteurs et s'expliquent par une plus grande sensibilité de la méthode. En plus on examina des frottis de sang de rats et de souris.

Zusammenfassung

Mit Hilfe eines Azofarbstoff-Verfahrens gelang es, in allen kernhaltigen Zellen von menschlichem Blut und Knochenmark saure Phosphatase nachzuweisen. Die Aktivität war in den *Granulozyten größer als in den Lymphozyten. Im Knochenmark* zeigten jugendliche Zellen eine größere Enzymaktivität als reife Zellen mit Ausnahme der Megakaryozyten, die sich entgegengesetzt verhielten. Die Ergebnisse weichen von denjenigen anderer Autoren ab, was wahrscheinlich auf die größere Empfindlichkeit der hier angewandten Methode zurückzuführen ist. Es wurden ferner Blutausstriche von Ratten und Mäusen untersucht.

References

1. Rabinovitch, M. and Andreucci, D.: A histochemical study of «Acid» and «Alkaline» phosphatase distribution in normal human bone marrow smears. Blood 4: 580 (1949).
2. Storti, E., Perugini, S., Rossi, V.: Quadro citotopochimico delle fosfatase nelle cellule. Medicina 3: 333 (1953).
3. Haight, W. F. and Rossiter, R. J.: Acid and alkaline phosphatase in white cells. Blood 5: 267 (1950).
4. Valentine, W. N. and Beck, W. S.: Biochemical studies on leukocytes. J. Lab. clin. Med. 38: 39 (1951).
5. Löffler, H. and Berghoff, W.: Eine Methode zum Nachweis von saurer Phosphatase in Ausstrichen. Klin. Wschr. 40: 363 (1962).
6. Pearse, A. G. E.: Histochemistry Theoretical and Applied, p. 882 (Churchill, London 1960).
7. Gomori, G.: The distribution of acid phosphatase in tissues under normal and under pathologic conditions. Arch. Path. 22: 189 (1941).

Authors' address: Dr. L. Rosenszajn, Dr. G. Marshak and Prof. Dr. P. Efrati, Laboratory for Blood Morphology and Cytology, Kaplan Hospital, Rehovoth (Israel).

XIIth Annual Colloquium, St. Jans Hospital, Bruges April 30 — Mai 3, 1964

Protides of the Biological Fluids

Topics: I. Phylogeny of proteins. – II. Tissue proteins. – III. Urinary glyco- and mucoproteins. – IV. Methods.

Round Tables. I. Tissue proteins. – II. Methods. – III. Proteins of diagnostic significance.

For applications and communications please supply to
Laboratory St. Jans Hospital, Bruges (Belgium)

Libri

Whitby and Britton. Disorders of the Blood. Churchill Ltd., London 1963. 9th ed. *by C. L. C. Britton*, 570 p. Price 100 sh.

This new edition of the well known textbook shows many changes in order to incorporate the remarkable progress made in many parts of haematology. Completely rewritten (and very well done) were the chapters on Cytochemistry of Haemopoiesis (by F. G. J. Hayhoe) and on *Blood Groups* and *Blood Transfusions* (by Geoffrey H. Tovey).

It is regrettable that the Index of Authors had to be omitted for lack of space. A number of typographical errors have crept into the text particularly regarding the names of the authors of quoted papers.

However these are minor blemishes. Undoubtedly the book with its great number of good black and white and color illustrations, its sound clinical judgement is a lucid, valuable and very readable presentation of todays haematology.

G. Rosenow, New York

John W. Harris. The Red Cell. Harvard University Press, Cambridge/Massachusetts 1963. 429 p. $ 5.75.

This is a very comprehensive monography of the red cell with over 2000 references of the literature. It was originally written for the use in courses of hematology for advanced students. The main emphasis is laid on physiology, biochemistry and pathology. Starting from this basis, short clinical descriptions are given concerning e.g. the Porphyrias, Sickle Cells Disease, Polycythemia, Anemias of the various types and etiology.

The physiological and pathological chapters are so complete and so well presented that many hematologists will highly value and use this monograph for its detailed information and references.

G. Rosenow, New York

John W. Harris and David L. Horrigan: **Case Development Problems in Hematology** Series 1. The Red Cell, Problems 1–8. Harvard University Press, Cambridge, Massachusetts 1963. 196 p. Price $ 3.25.

The little book supplements the text material of the "Red Cell" (reviewed above). It gives case reports for the student as a learning device in order to test his diagnostic and physiological knowledge by asking questions and providing the correct answers.

O. Rosanow, New York

Zyto- und Histochemie in der Haematologie. Neuntes Freiburger Symposion an der medizinischen Universitätsklinik vom 25. bis 27. Oktober 1962. Hgb. von *Hans Vorder.* Zugleich Symposion der Gesellschaft Deutscher Hämatologen. Springer-Verlag, Berlin 1963. 000 S., 00 Abb., 00 Tab. Preis DM 98.

Referate und Diskussionsbeiträge dieses Symposiums werden in extenso wiedergegeben. Die behandelten Themata lauten: 1. Nukleinsäuren und Proteine. 2. Enzyme. 3. Polysaccharide und Lipide. 4. Eisen und andere Mineralien. Die Behandlung dieses neuen und schnell sich ausweitenden Gebietes der modernen Haematologie ist sehr umfassend. Inhaltsverzeichnis, Sachverzeichnis und Darstellung sind vortrefflich. Der interessierte Forscher und Arzt findet darin nicht nur die neuesten Ergebnisse, sondern kann das Buch als wertvolle Informationsquelle über hämatologische Zyto- und Histochemie benutzen.

P. Frick, Zürich

Acta haemat. 30: 319–325 (1963)

From Medical Department B, Bispebjerg Hospital, Copenhagen

Pure Red Cell Anaemia and Thymoma*

By Stig Bryde Andersen and Jorgen Ladefoged

Not more than about 75 cases of pure red cell anaemia have been reported since Kaznelson (1) first described this disorder in 1922 Matras and Priesel (2) reported in 1928 a case history of a patient with this anaemia and thymona, and since then the association of thymoma and pure red cell anaemia has been reported in 43 cases (3) a coincidence far exceeding a chance relationship No aetiological explanation has been offered for this relationship, because the function of the thymus has been obscure. Recent experiments have shown that the thymus plays an important role in the development of the immunological functions of the organism (4) The purpose of this paper is to summarize the syndrome, to report another case and finally in the light of newly acquired knowledge on the function of the thymus, to give an obvious explanation of the pathogenesis of the syndrome.

The clinical picture and the course of the disease show a clinical and pathological entity Based on 32 cases, 29 from the literature and 3 of their own, Havard and Scott (5) have described the syndrome in detail. The following survey is based on 44 cases (3) and is in good agreement with that of Havard and Scott

The frequency of the syndrome is not known exactly but judging from the literature pure red cell anaemia seems to be found in about 5 / of all patients with thymoma (6 7) and thymoma in more than half the patients with pure red cell anaemia (8, 6) Pure red cell anaemia without thymoma, has been described only in about 30 cases. The tumour is benign but local invasion of the pleura may occur The microscopical appearance is very inconsistent, but two elements are always recognized, namely epitheloid

* This work was supported by grant from King Christian Xth Foundation.

cells ("spindle cells") and lymphocytes ("thymocytes"). No relationship has been found between the histology of the tumour and the symptomatology of the disease. The thymoma may appear up till 20 years before the anaemia, or it may be recognized during the course of the anaemia, or perhaps not until the autopsy. The anaemia may even develop several years after radical excision of the tumour.

Pure red cell anaemia is characterized by severe anaemia, absence of reticulocytes in the peripheral blood and no alterations in leucocytes and platelets. Bone marrow aspirations show selective aplasia or hypoplasia of erythropoiesis while myelo- and thrombocytopoiesis is normal. Some patients may develop pancytopenia. The bone marrow in 6 patients out of 44 was aplastic but the anaemia in these patients was initially more outstanding than thrombocytopenia and granulocytopenia. The anaemia may at the beginning appear as a typical autoimmune haemolytical anaemia with positive Coombs test (4 patients of 44) and then later appear as pure red cell anaemia with negative Coombs test. The survival of erythrocytes measured with ^{51}Cr-labelling is normal or perhaps slightly reduced. Some patients may develop agammaglobulinaemia (4 out of 44 patients) and some may have myasthenia gravis (5 out of 44).

The symptoms are due to the anaemia, even large thymomas only rarely give symptoms. No enlargement of liver, spleen or lymph nodes is found. Terminally haemorrhagic diathesis and frequent infections may be seen.

Repeated blood transfusion is the only effective treatment. Only few patients improved after thymectomy (4 out of 18). Irradiation of the tumour is without effect. Splenectomy only has a transitional effect in the patients with autoimmune haemolytic anaemia. Treatment with ACTH, corticosteroids and testosteron has been used extensively but with doubtful effect.

The prognosis is bad but the patients may be kept in good condition for years by means of blood transfusions. Spontaneous or induced remission is seen in about one fourth of the patients. After the diagnosis of pure red cell anaemia has been made the patients life expectation varies from a few months to 20 years, with an average of two to three years. The cause of death usually is infection (due to agranulocytosis, agammaglobulinaemia, treatment with steroids or blood transfusions), progressive anaemia or cardial complications. Autopsy has not been able to explain the syndrome,

the only constant finding is haemosiderosis caused by the great number of transfusions given.

Case History

A man, born 1893, was at the age of 50 admitted on account of diarrhoea and severe anaemia (haemoglobin 4.8 g/100 ml). Exploratory laparotomy performed on suspicion of intestinal tumour, only showed slightly enlarged mesenteric lymph nodes (Microscopical examination Hyperplasia of the reticuloendothelial system). The nature of the anaemia was not disclosed and it disappeared spontaneously after some months. At the age of 56 a tumour in the anterior mediastinum was found by routine chest roentgenogram. A 10 × 10 cm tumour was removed. Microscopical examination (Dr CHARLES JOHANSEN) A connective tissue membrane was found around the tumour. Inside the membrane was found net of light, clear, slightly irregular cells with large light nuclei. Among the net were round cells, a little greater than normal lymphocytes, with rather large nucleus sometimes containing nucleolus. No atypical nuclei were found and only few mitoses. Only few HASSALL's corpuscles could be identified. No breaking through *of the connective tissue membrane was found. Diagnosis: Thymoma, probably malignant.* Since the tumour was considered to be malignant 2850 was given locally post operatively The peripheral blood was found to be normal during the admission.

At the age of 58 the patient was admitted on account of diarrhoea and severe anaemia (haemoglobin 4.5 g/100 ml). Bone marrow aspiration showed complete absence of erythroblasts, but normal granulo- and thrombocytopoiesis. Thrombocytes and leucocytes in the peripheral blood were found to be normal, but reticulocytes were below 0.1 ‰. A slightly positive COOMBS test was found on several occasions but otherwise there were no signs of haemolytic anaemia. No abnormal antibodies were found in the serum. The patient was treated with about 50 blood transfusions (500 ml) in the next three years. Then the patient was treated with ACTH (15 × 20 units of Acton Prolongatum(R)) followed by complete remission. Four years later 65 years old, the patient again was admitted with diarrhoea and severe anaemia (haemoglobin 4.4 g/100 ml) and haematological findings *identical with the ones found seven years earlier.* Biopsy from the iliac crest did not show myelofibrosis and COOMBS test was positive once, but otherwise negative. Half-life of Cr^{51}-labelled erythrocytes was 22 days (normal values 25–30 days) and no accumulation of radioactivity was found in liver and spleen. The osmotic resistance of the erythrocytes was normal. Electrophoresis of serum was normal apart from slightly increased gamma globulin. Sedimentation rate was 73 mm/hours. Roentgenological examination of the gastrointestinal tract and chest was normal. No blood was *found in feces, but from 30–70g lipids/24 hours (normals below 5 g/24 hours).* Absorption tests for vitamin A, d-xylose and vitamin B_{12} (SCHILLING's test) showed reduced values. Concentration of serum iron and serum B_{12} was normal. Neurological examination showed that the patient did not have myasthenia gravis. Liver and kidney functions were normal, serum bilirubin was 0.6 mg/100 ml.

During the next two years the patient was treated with blood transfusions. Treatment with ACTH, steroids, vitamins, iron and vitamin B_{12} was tried but without effect. Gluten free diet did not change the steatorrhoea.

At the age of 67 spontaneous remission of the anaemia occurred, lasting until the patient died, 69 years old. The patient did not have thrombocytopenia, granulocytopenia, or agammaglobulinaemia. Autopsy showed severe haemosiderosis and bronchopneumonia on both sides. No thymic tissue could be found and the bone marrow was normal. The remaining internal organs were normal, including the spleen, lymph nodes and gastrointestinal tract.

Discussion

The association of two so rare disorders as thymic tumours and pure red cell anaemia indicates an aetiological relationship. Also other disorders are associated with thymomas as for instance myasthenia gravis agammaglobulinaemia and CUSHING's syndroma. The combination of myasthenia gravis and thymoma is relatively frequent and in recent years suggestive evidence has been presented that myasthenia gravis is an autoimmune disease (9 10 11) The association of thymoma and other disorders presumably of autoimmune aetiology has also been reported (12 13) and based on clinical and experimental studies BURNET AND MACKAY (14) have suggested that structural changes of the thymus are found generally in autoimmune disease. It is therefore tempting to presume that pure red cell anaemia, a disorder certainly associated with structural changes of the thymus, is an autoimmune disease.

Provided that autoimmune mechanism is responsible for the development of pure red cell anaemia, then the autoantibody can not be directed against the erythrocytes, because haemolytic anaemia rarely is found in these patients. The autoantibodies therefore have to be directed against either the erythroblasts, the nuclei of the erythroblast or some factor governing the production of erythrocytes, for instance erythropoietin. It is well known that immunologic or allergic mechanisms are able to produce a selective erythroblastic aplasia in the bone marrow for example in typical autoimmune haemolytic anaemias (15–21) following iso-immunization by blood group A (22) or rhesus-isoimmunization (23) and in the course of diseases associated with autoimmune phenomenons, for instance leukaemia (24) or lymphosarcoma (6) SCHOOLEY AND GARCIA (25) have been able to reduce the erythropoiesis in mice by means of an antibody against erythropoietin suggesting that an autoantibody against erythropoietin might be able to produce pure red cell anaemia in man. The considerations presented here are mostly hypothetical. On the other hand suggestive evidence has been presented that pure red cell anaemia actually is an autoimmune condition, perhaps directed against erythropoietin. This taken together with the fact that previous studies have not been able to reveal the true nature of this disease may give the present explanations value. Thus it has not been possible to demonstrate a humoral

factor in the development of pure red cell anaemia (6 26 27) and it is unlikely that the tumour should be directly responsible for the anaemia because extirpation of the thymoma will rarely cure the disease. Pure red cell anaemia may even develop several years after the extirpation of the tumour But neither is antibody production in the tumour able to explain the development of an autoimmune disease for the same reasons. However according to the hypothesis of BURNET (28) on the function of thymus these inconsistencies may be explained. BURNET considers the thymus to have two functions. The first is concerned with the differentiation of lymphocytic cell lines for specific immunologic functions and the second is concerned with immunologic homeostatic control. The development of autoimmune disease may in that case be due either to reduction of the normal thymic immunologic control or to development of cell clones resistant to the *normal* thymic control. If both mechanisms are responsible for the development of autoimmune disease, then it is understandable why thymectomy sometimes will and sometimes will not cure pure red cell anaemia. If, for example, the autoimmune disorder is due to development of "forbidden clones" in some part of the thymus then thymectomy should be able to cure the autoimmune disease. But if the autoimmune disease is caused by a reduction of the normal homeostatic control in consequence of damage to the thymus then, of course, thymectomy should not improve the autoimmune disorder

Summary

This is report of case history of patient with *pure red cell anaemia* and thymoma and also survey of the clinical picture and the course of the disease based on 43 cases from the literature. In the light of newly acquired knowledge on the function of the thymus the pathogenesis of the syndrome is explained as an autoimmune disorder the autoantibody of which is directed against erythroblasts or against some factor governing erythropoiesis, for example erythropoietin.

Résumé

L auteur rapporte l'observation d'un patient atteint d'une anémie aplastique avec thymome et rappelle le tableau clinique et l'évolution de la maladie d'après 43 cas déjà publiés. A la lumière des récentes notions sur la fonction du thymus, il semble que la pathogénèse de l'affection relève d'un processus d'autoimmunisation où l'anticorps est dirigé contre les érythroblastes ou contre un facteur gouvernant l'érythropoïèse, par exemple l'érythropoïétine.

Zusammenfassung

Es wird über einen Fall von Anämie bei Thymom berichtet. Auf Grund von 43 in der Literatur beschriebenen Fällen wird eine Übersicht über das klinische Bild und den Verlauf der Krankheit gegeben. Im Lichte neuerer Erkenntnisse über die Thymusfunktion wird die Pathogenese des Syndroms auf eine Autoimmunkrankheit zurückgeführt, wobei der Antikörper gegen die Erythroblasten oder gegen einen erythropoetischen Faktor wie z.B. das Erythropoietin, gerichtet ist.

References

1. Kaznelson, P Zur Entstehung der Blutplättchen. Verh. dtsch. Ges. inn. Med. *34* 557 (1922)
2. Mathias, A. and Prinzel, A.: Über einige Gewächse des Thymus. Beitr path. Anat. *80*: 270 (1928)
3. Ladefoged, J. and Andersen, S. B.: Thymoma and "pure red-cell" anaemia. Ugeskr Laeger (in press).
4. Miller, J. A. F P: Immunological function of the thymus. Lancet *ii*. 748 (1961).
5 Havard, C. W H. and Scott R. B.: Thymic tumour and erythroblastic aplasia. Brit. J. Haemat. *6*: 178 (1960).
6. Jacobs, E. M. Hutter, R. V P; Pool, J L. and Ley A. B. Benign thymoma and selective erythroid plasia of the bone marrow Cancer *12*. 47 (1959).
7 Bernatz, P E. Harrison, E. G. and Clagett O. T Thymoma, a clinopathologic study J thor cardiovasc. Surg. *42*: 424 (1961)
8. Tsai, S. Y and Levin, W C. Chronic erythrocytic hypoplasia; review of literature and report of a case. Amer J Med. *22*: 322 (1957)
9. Simpson, J A. Myasthenia gravis A new hypothesis. Scot. med. J. *5*. 419 (1960).
10. Strauss, A. J L. Seegal, B. C.; Hsu, J C. Burkholder, P M. Nastuk, W L. and Osserman K. E. Immunofluorescence demonstration of muscle binding complement fixing serum globulin fraction in myastenia gravis. Proc. Soc. exp. Biol., N Y *105*. 184 (1960)
11 White, R. G. and Marshall, A. H. E. The auto-immune response in myastenia gravis. Lancet *ii*. 120 (1962)
12. Funkhouser, J W Thymoma associated with myocarditis and the L. E.-cell phenomenon. New Engl. J Med. *264* 34 (1961).
13. Smithers, D W Tumours of the thyroid gland in relation to some general concepts of neoplasia. J Fac. Radiol. *10*: 3 (1959)
14 Burnet, F M. and Mackay I. R. Lymphoepithelial structure and autoimmune disease. Lancet *ii*. 1030 (1962)
15. David, L. J Kennedy A. C.; Baikie, A. G. and Brown, A.: Haemolytic anaemias of various types treated with ACTH and cortisone. Glasg. med. J. *33*: 263 (1952).
16. Linke, A. Klinische und experimentelle Beobachtungen über aplastische Krisen der Erythropoese bei haemolytischen Anämien. Verh. dtsch. Ges. inn. Med. *58*: 724 (1952)
17 Ackermann, G and Dameshek, W Splenectomy for "pure red-cell" hypoplastic anemia associated with autoimmune hemolytic disease; report of case. New Engl. J Med. *251* 1044 (1954).
18. Seip M. Aplastic crisis in case of immunohemolytic anem
153: 157 (1955).
19 Bove, J R. Combined erythoid hyperplasia and symp
New Engl. J Med. *255*: 135 (1956).

20. BURSTON, J.; HUSAIN, O. A. N.; HUTT, M. S. R. AND TANNER, E. I. Two cases of auto-immune haemolysis and aplasia. Brit. med. J. i: 83 (1959).
21. MEYER, L. M. AND BERTCHER, R. W. Acquired hemolytic anemia and transient erythroid hypoplasia of bone marrow. Amer. J. Med. 28: 606 (1960).
22. SMITH, C. H. Chronic congenital aregenerative anemia associated with iso-immunization by blood group A. Blood 4: 697 (1949).
23. GIBLETT, E. R., VARELA, J. E. AND FINCH, C. A. Damage of the bone marrow due to Rh antibody. Pediatrics 17: 37 (1956).
24. SOUTTER, L. AND EMERSON, C. P. Elective thymectomy in the treatment of aregenerative anaemia associated with monocytic leukaemia. Amer. J. Med. 28: 609 (1960).
25. SCHOOLEY, J. C. AND GARCIA, J. F. Immunochemical studies of human urinary erythropoietin. Proc. Soc. exp. Biol., N. Y. 109: 325 (1962).
26. RAMOS, A. J. AND LOEB, V. Diagnostic problems. Presentation of a case and comments. J. amer. med. Ass. 160: 1317 (1956).
27. PARR, E. H. O.; KILPATRICK, G. S. AND HARDISTY, R. M. Red-cell aplasia and benign thymoma. Studies on a case responding to prednisone. Brit. med. J. i: 1154 (1959).
28. BURNET, F. M. The immunological significance of the thymus. Austr. Ann. Med. 11: 79 (1962).

Authors' address: Dr. B. R. Andersen and Dr. J. Ladefoged, Dept. of Clinical Physiology, Glostrup Hosp., Copenhagen (Denmark).

Acta haemat. 30, 326–333 (1963)

From the Department of Hematology, Walter Reed Army Institute of Research, Washington, D. C.

Increased Urea Solubility of the Blood Clot in Chronic Granulocytic Leukemia and its Relation to the Abnormal Thrombelastogram

HARVEY J. WEISS

WINCKELMAN, HÖRDER AND PILEGGI have described an abnormal thrombelastographic pattern in patients with chronic granulocytic leukemia (CGL) (1, 2). The clot appears to become detached from the wall of the instrument's cuvette, resulting in a pattern resembling fibrinolysis, which they called the "step phenomenon" (SF). WINCKELMAN et al. attribute the phenomenon to a proteolytic action of the leukocytes at the edges of the clot. MARCHAL and co-workers thought of platelet abnormalities (3, 4).

The abnormal thrombelastogram (TEG) appears to be the first consistent abnormality pertaining to hemostasis which has been found in CGL. Although these patients frequently have a bleeding tendency, clotting tests have not revealed any well defined abnormality. The abnormal TEG suggests that perhaps the clot is structurally defective. If, for example, it failed to adhere to wound edges or was easily dislodged by mechanical or retractile forces, impaired hemostasis might result.

The present report presents data which show that the blood clot in patients with chronic granulocytic leukemia is abnormally soluble in 5 M urea.

Methods

Thrombelastogram of blood and plasma was obtained by the method of HARTERT (5). The solubility of blood clots in 5 M urea was determined as follows. 0.5 ml of clotted whole blood or plasma was incubated in glass tubes for 4 hours at 37° C. The serum was drained off and 5 ml of 30% (W/V) urea added. The tubes were stoppered, vertically rotated at 4 RPM and observed for lysis. As controls, blood clots were rotated with distilled water or phosphate buffer, pH = 7.2.

Tensile strength was determined on whole blood clots which had been formed in lucite mold, as shown in fig. 1a. The dumbbell-shaped mold was formed by fitting to-

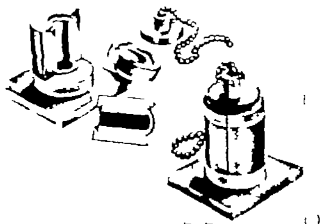
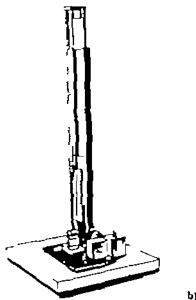


Fig. 1 Apparatus for measuring tensile strength of blood clots) detailed drawing of lucite mold in which blood clots are formed; the hollow inner cylinder is 1.5 cm long and 0.33 sq. cm in cross sectional area; b) assembled machine, showing lucite mold attached to Jolly balance.



b)

gether four pieces of lucite (identical sides and two identical ends. Its inner surface was coated with paraffin prior to use. Whole blood was poured directly into the mold and allowed to clot in 37 C incubator for 1 hour. The mold was then affixed to the base of specially constructed Jolly balance (fig. 1b) and the sides removed, leaving the clot suspended between the base and the top. The spring of the balance was stretched by motor driven shaf at the rate of 3.3 mm per sec and the tensile strenght of the clot

was determined from the net excursion of the spring at the point where the clot broke. In almost all instances, break occurred at either end, rarely in the middle. Tensile strength of normal clots in this apparatus was 2.8–6.1 gm. These values are similar to the values of 4.48 (2.0–7.3) reported by MACFARLANE AND TOMLINSON (6) for whole blood using clots of similar cross sectional diameter

The velocity of clot retraction in glass tubes was determined in 15 ml graduated conical centrifuge tubes. Five ml of blood was added and the tube stoppered with rubber stopper through which metal hook was inserted. The tube was inverted and the blood allowed to clot around the hook. After clotting, the tube was again inverted to the upright position and placed in 37 C incubator. At various intervals, the amount of serum expressed was read directly from the scale on the tube.

Results

Thrombelastogram The step phenomenon (SF) was observed in 9 out of 10 patients with chronic granulocytic leukemia during some phase of their illness (fig 2a, b) The phenomenon usually disappeared as the patient's white count approached normal, but was occasionally still seen with a count of 35 000. The only untreated patient whose blood did not demonstrate this phenomenon had a leukocyte count of 65 000 and a differential count which

Table I
Solubility of blood clots in 5 M urea.

	Time (hours)							
	0	16	24	48	72	WBC	[illegible]	Step phenomenon (SF) on TEG
Normal Subjects								
No. 1	3+	2+	2+	1+	1+			
2	3+	3+	3+	2+	1+			
3	3+	3+	3+	2+	1+			
4	3+	3+	2+	2+	1+			
	3+	3+	2+	2+	1+			
6	3+	3+	2+	1+	0			
Patients with chronic granulocytic leukemia								
No. 1	3+	+	0	0	0	40,000	Yes	Yes
2	3+	0	0	0	0	35,000	Yes	Yes
3	3+	2+	±	±	0	40,000	Yes	No
4	3+	3+	3+	+	±	68,000	Yes	No
	3+	3+	2+	0	0	82,000	Yes	Yes
6	3+	3+	3+	3+	3+	15,000	Yes	No
7	3+	1+	0	0	0	350,000	No	Yes
8	3+	2+	1+	0	0	75,000	No	Yes
9	3+	0	0	0	0	120 000	No	Yes
10	3+	0	0	0	0	80 000	Yes	Yes

The size of each blood clot is graded from 0 (complete dissolution) to 3+

showed mainly mature neutrophiles or band forms. When the leukocytes of whole blood were removed by centrifugation, the SF was no longer observed in the supernatant plasma, as described by WINCKELMANN AND HÖRDER (2) When washed leukemic leukocytes were added to normal blood a slight SF resulted, but only at very high concentration (280 000 per mm³) The addition of washed platelets (140 000 per mm³) had no effect.

Urea Solubility The whole blood clots of the patients dissolved more rapidly in 5 M urea than did normal clots (table I) The latter were not usually completely dissolved even after 72 hours of incubation. By contrast, in 5 out of 10 patients with CGL, the clots were completely dissolved after 24 hours and in 2 others they were dissolved after 48 hours. Abnormal clot lysis was also observed in 3.3 M urea, *but not in concentrations below this. When the urea* was removed by prolonged dialysis against saline the clot did not reappear This is in contrast to the situation when fibrin stabilizing factor or calcium is missing as described by LORAND (7) In three

Table II

Tensile strength of blood clots.

Patients with Chronic Granulocytic Leukemia	Tensile Strength in Grams (Normal 2.8–6.3)
No. 2	6.7
7	8.5
8	6.2
9	7.6
10	6.6

patients, all under therapy a clot was still *present after 48 hours* and in two of these at 72 hours. There was excellent correlation between the urea solubility of the clot and the TEC In the three patients whose clots had not dissolved after 48 hours, the SF was not present. *The abnormal urea solubility like the SF required the presence of leukocytes or platelets or both.* Thus, if blood from a patient with CGL was centrifuged at 1 000 RPM *for 4 minutes* and the supernatant, cell rich plasma was allowed to clot it dissolved readily in 5 M urea, similar to the clotted whole blood. If the blood was centrifuged further at 3 000 RPM for 10 minutes, removing all white cells and most platelets, the clot from the supernatant plasma was then insoluble in 5 M urea.

Effect of Reduced Glutathione The addition of reduced glutathione to whole blood, final concentration 0 01 M, eliminated the SF in

reappear when the urea is removed by dialysis. By contrast, leukemic clots do not, suggesting that true proteolysis has occurred. Such potentiation by urea and urea derivatives has been noted for other proteolytic systems (11, 12). In addition, the leukemic clots, unlike those deficient in FSF, have a normal tensile strength. Thus, it is not clear whether the increased urea solubility of leukemic clots reflects a structural weakness. Whatever the cause of the increased solubility, this abnormality appears to correlate with the presence of the step-phenomenon on the TEG. Both phenomena require the presence of platelets or leukocytes or both. Differential centrifugation studies suggest that the leukocytes are probably the main factor, although attempts to reproduce the SF with isolated washed cells yielded inconclusive results.

Reduced glutathione, in concentration 0.01 M, consistently corrected the patients' abnormal TEG; similarly, it also retarded the abnormally rapid retraction from glass surfaces. The concentration of glutathione required to inhibit the SF is similar to that which is effective against certain fibrinolytic systems (13). If, as WINCKELMAN et al. believe, the abnormality is due to a leukocyte protease, glutathione may act by inhibiting it. Unlike its effect on the TEG, glutathione was completely ineffective in correcting the abnormal urea solubility. This suggests that the increased urea solubility is not due to inhibition of FSF, since glutathione can completely substitute for this factor (10).

Finally, are the abnormal TEG and the urea solubility of the clot related in any way to the abnormal hemostasis in CGL? DE NICOLA has likened the clot stressing forces in the TEG cuvette to those acting at the edges of a wound (14). Do the clots in patients with CGL exhibit the step phenomenon *in vivo*? That is, do they fail to adhere adequately to the edges of a wound and become easily detached, as in the TEG cuvette? Experiments on the adhesion of blood clots to nonviable tissue did not suggest that the clots from these patients were any less adhesive than those of normal subjects.

Summary

The whole blood clots of patients with chronic granulocytic leukemia are more soluble in 5 M urea than are normal blood clots. This finding correlates with the abnormal thrombelastogram (step-phenomenon) which is seen in this disorder. Like the step-phenomenon, the abnormal urea solubility requires the presence of white cells or platelets or both. The abnormal TEG, but not the increased urea solubility, can be corrected by reduced glutathione, 0.01 M. The increased urea solubility is probably due to potentiation of proteolysis rather than an inhibition of fibrin stabilizing factor.

Résumé

Le caillot de sang total des patients atteints de leucémie myéloïde chronique est plus soluble dans l'urée 5 M que les caillots de sujets témoins. Ce fait concorde avec l'anomalie thrombélastographique (»steps«) que l'on décrit dans cette affection. A l'instar de ce phénomène du »steps«, la solubilité anormale à l'urée requiert la présence des leucocytes ou des plaquettes, voire des deux. Le glutathion réduit 0,01 M peut corriger le TEG mais non la solubilité excessive à l'urée. Cette dernière est due probablement plus à une potentialisation de la protéolyse qu'à une inhibition du facteur stabilisateur de la fibrine.

Zusammenfassung

Blutgerinnsel von Patienten mit chronischer myeloischer Leukämie sind in 5 M Harnstoff leichter löslich als normale Blutgerinnsel. Dieser Befund steht in Beziehung zum abnormen Thrombelastogramm (Stufenphänomen) bei dieser Krankheit. Wie das Stufenphänomen hat die abnorme Harnstofflöslichkeit die Gegenwart von Leukocyten oder Thrombocyten oder von beiden zur Voraussetzung. Das abnorme TEG, jedoch *nicht die gesteigerte Harnstofflöslichkeit*, lassen sich durch 0,01 M *reduziertes* Glutathion korrigieren. Die Steigerung der Harnstofflöslichkeit ist wahrscheinlich eher durch eine Verstärkung der Proteolyse, als durch eine Hemmung des fibrinstabilisierenden Faktors bedingt.

References

1. Hörder, M. H. und Pilgrim, J. Beeinflussung der Haftfähigkeit des Thrombus durch Leukocyten der chronisch leukämischen Myelose. Acta haemat. *17* 111–115 (1957).
2. Wendelmann, G. und Hörder, M. H. Veränderung der Gerinnselhaftfähigkeit durch Leukocyten der myeloischen Reihe. Acta haemat. *18*: 261 (1957).
3. Marchal, G.; Leroux, M. E. et Samama, M. Les anomalies thrombodynamographiques dans les leucémies myéloïdes. Sang. *29*: 265 (1958).
4. Marchal, G.; Leroux, M. E.; Duhamel, G. et Samama, M. Aspects thrombodynamographiques de l'hyperplaquettose dans les leucémies myéloïdes et dans les thrombocythémies hémorragiques. Sang. *29*: 666 (1958).
5. Hartert, H. Blutgerinnungsstudien mit der Thrombelastographie, einem neuen Untersuchungsverfahren. Klin. Wschr. *26*: 577 (1948).
6. Macfarlane, R. G. and Tomlinson, A. H. An apparatus for measuring the tensile strength of blood clots. J. clin. Path. *14*: 320 (1961).
7. Lorand, L. Fibrin clots. Nature *166*: 694 (1950).
8. Laki, K. and Lorand, L. On the solubility of fibrin clots. Science *108*: 280 (1948).
9. Ferry, J. D.; Miller, M. and Shulman, S. The conversion of fibrinogen to fibrin. VII. Rigidity and stress relaxation of fibrin clots; effect of calcium. Arch. biochem. biophys. *34*: 424 (1951).
10. Loewy, A. G. and Edsall, J. T. Studies on the formation of urea-insoluble fibrin. J. biol. chem. *211*: 829 (1954).
11. Jürgens, L. und Wörlich, E. Über Fibrinogenolyse unter dem Einfluß hydrotroper Substanzen. Biochem. Z. *330*: 312 (1958).
12. von Kaulla, K. N. and Smith, R. L. Urea derivatives as fibrinolysis promoting agents. Proc. Soc. exp. Biol., N. Y. *106*: 530 (1961).
13. Mootse, G. and Clifton, E. The effect of *cysteine and mercaptoethanol* on plasmin. Amer. J. Cardiol. *6*: 399 (1960).
14. de Nicola, P. Thrombelastography. p. 18 (Charles C Thomas, Springfield, Ill. 1957).

Author's address: Dr. H. J. Weiss, Dept. of Medicine, New York University School of Med., 550 First Avenue, New York, N.Y. (USA).

Acta haemat. 30: 334–339 (1963).

Aus der II. Medizinischen Universitätsklinik in Wien
(Vorstand: Prof. Dr. K. Fellinger)

Esterase- und Lipase-Aktivität in den weißen Blutzellen

Von H. Braunsteiner, F. Dienstl, S. Sailer und F. Sandhofer

In den letzten Jahren hat der cytochemische Nachweis von unspezifischen Esterasen in den Leukozyten zunehmendes Interesse gefunden. Als Substrate wurden vor allem Alpha-Naphthylacetat, Naphthol-AS-Acetat und Naphthol-AS-D-Acetat verwendet (9). Übereinstimmend konnte eine schwache Esteraseaktivität in den reifen Granulozyten, eine stärkere in den Myelo- und Promyelozyten und eine sehr starke Aktivität in den Monozyten und Histiozyten nachgewiesen werden. Lymphozyten zeigten dagegen keine oder nur eine sehr schwache Aktivität. Der Ausfall der Reaktion ist weitgehend unspezifisch; ob damit Lipasen erfaßt werden, ist ungewiß.

Biochemische Untersuchungen sind bisher erst spärlich durchgeführt worden. Hardin et al. (6) haben eine deutliche Esteraseaktivität mit Beta-Naphtholacetat als Substrat sowohl in Granulozyten als auch in Lymphozyten kolorimetrisch nachgewiesen. Die Spaltung von Beta-Naphthol-Laureat in Granulozyten und Lymphozyten wurde als Hinweis für das Vorhandensein einer Lipase in den genannten Zellen interpretiert. Zu ähnlichen Ergebnissen kamen Frei et al. (5). Die Spezifität dieses Substrates muß jedoch angezweifelt werden. Als Substrat zur Messung einer Lipaseaktivität müssen Emulsionen von Triglyceriden langkettiger (1) Fettsäuren verwendet werden. Eine weitere Bedingung für eine exakte Lipasebestimmung ist die Wasserunlöslichkeit des verwendeten Substrates (11).

Zum Lipasenachweis in Leukozyten liegen nur Arbeiten von Bergel (2) sowie Nees (10) vor, in denen die Andauung von Bienenwachs und Schweinefett durch Eiter untersucht wurde. Den

ersten bisher verwertbaren Lipasenachweis in Leukozyten lieferten Izak und de Vries (7)

Das Ziel unserer Untersuchungen bestand einerseits im Nachweis einer etwaigen Lipase in den weißen Blutzellen, andererseits sollte die hydrolytische Spaltung von Triglyceriden kurzkettiger Fettsäuren (Tributyrin, Tricaproin und Tricaprylin) biochemisch gemessen und mit dem Ausfall cytochemischer Untersuchungen unter Verwendung von Naphthol AS-D-Acetat als Substrat in Vergleich gebracht werden.

Methode

Die untersuchten weißen Blutzellen stammen von Patienten, die sich in stationärer Behandlung der Klinik befanden.

Anreicherung aus peripherem Blut. 30 ml durch Venenpunktion frisch gewonnenes Vollblut werden in einem silikonisierten Zentrifugenglas mit 15 ml einer 3%igen Dextranlösung (Dextran 250, η = 0,42, Mw = 267 000, Mn = 139 000, Fa. Knoll AG, Ludwigshafen/Rhein, in 0,9%iger NaCl-Lösung) 10 000 E Streptokinase, 2500 E Streptodornase (Varidase, Fa. Lederle, American Cyanamide Comp.) und 250 I. U Liquemin Roche vorsichtig gemischt und 30 Minuten bei Zimmertemperatur stehen gelassen. Während dieser Zeit setzen sich die Erythrocyten am Boden ab. Der Überstand wird vorsichtig abgegossen und 5 Minuten bei 140 × g zentrifugiert. Der Überstand wird verworfen, und die verbliebenen Zellen werden durch vorsichtiges Schütteln in 40 ml 0,9%iger NaCl-Lösung aufgeschwemmt und neuerlich auf die angegebene Art zentrifugiert. Dieser Waschvorgang wird zweimal durchgeführt. Zur Entfernung der in der Zellsuspension noch verbliebenen Erythrocyten werden die Zellen mit 3 Teilen destilliertem Wasser genau 30 Sekunden geschüttelt und dann mit 1 Teil 0,6 m KCl-Lösung versetzt. Diese Suspension wird neuerlich bei 140 × g 5 Minuten zentrifugiert und der nun rötlich gefärbte Überstand abgegossen. Die Zellen werden dann entsprechend der gewünschten Zellzahl mit Verdünnungsflüssigkeit versetzt und in der Zählkammer nach Bürker Türk zweimal gezählt.

Gewinnung von Lymphocyten aus Lymphknoten und Milz: Das frisch entnommene Organmaterial wird in Hanks'scher Lösung mit Pinzette und Schere in möglichst kleine Stückchen zerkleinert, bis es annähernd breiartige Konsistenz hat. Zur Abtrennung gröberer Organbestandteile wird der Brei durch feinmaschige Gaze gepreßt. Die Zellen werden in Hanks'scher Lösung aufgeschwemmt, wie oben angegeben zweimal gewaschen und in der Zählkammer nach Bürker-Türk doppelt gezählt.

Bestimmung der Esterase-Aktivität: Als Substrate werden Tributyrin (Glycerin-tri-n-Butyrat der Fa. Fluka AG, Buchs SG), Tricaproin und Tricaprylin (California Corporation for Biochemical Research, Los Angeles 63, Cal.) verwendet.

Ansatz In den Haupttrog der Warburggefäße werden 0,1 ml Substrat und 1,9 ml der zu untersuchenden Zellen in einer Gesamtzahl von etwa 1×10^{8} pro Ansatz, aufgeschwemmt in 0,9%iger NaCl-Lösung, die als Puffersubstanz $NaHCO_3$ in einer Konzentration von 0,024 m enthält, eingepippetiert. Das Gesamtvolumen beträgt demnach 2,0 ml, pH 7 4. Für jede Probe werden je zwei Leerwerte mitbestimmt 0,1 ml Substrat + 1,9 ml $NaHCO_3$-Puffer in 0,9%iger NaCl-Lösung, bzw 1,9 ml der Zellsuspension, die mit Puffer auf 2,0 ml ergänzt wird. Gasphase 95% N_2, 5% CO_2. Die Gefäße werden in der Warburg-Apparatur bei 37 C geschüttelt, die manometrische Ablesung erfolgt alle 5 Minuten. Die Reaktion verläuft nach Einstellung des Temperaturgleichgewichtes unter den gegebenen Bedingungen über mindestens 1 Stunde linear. Die Akti-

vitäten werden unter Berücksichtigung der Leerwerte berechnet und in Internationalen Einheiten (I. U. = Mikromol freigesetzte Fettsäuren/10^{10} Zellen/Minute) angegeben.

Bestimmung der Lipase-Aktivität: Als Substrat dient eine Emulsion von Kokosfett (»Ceres« der Fa. Kunerol GmbH) in Nüchternplasma, die mittels Ultra-Turrax (Fa. Janke und Kunkel K.G., Staufen i. Br.) hergestellt wird.

Ansatz: 0,4 ml der 30%igen Emulsion, 0,1 ml 0,4 m Tris, 0,1 ml 5% Glukoselösung, 1,4 ml einer Suspension der zu untersuchenden Zellen in 0,9% NaCl-Lösung. Die Gesamtzahl der Zellen beträgt pro Ansatz $1-2 \times 10^{8}$, pH 8,2. Leerwert: Anstelle der Zellsuspension werden 1,4 ml 0,9% NaCl-Lösung eingesetzt. Jeder Ansatz wird in dreifacher Menge hergestellt; davon werden in 2 ml die freien Fettsäuren nach der von Dole und Meinertz (4) angegebenen Mikrotitrationsmethode sofort bestimmt, 2,0 ml derselben Ansätze werden im Wasserbad bei 37° C unter Luft eine Stunde geschüttelt und anschließend die freien Fettsäuren auf dieselbe Weise bestimmt. In vorhergehenden Untersuchungen wurde festgestellt, daß die Reaktion innerhalb dieser Zeit unter diesen Versuchsbedingungen linear verläuft.

Die lipolytische Aktivität wird aus der Differenz der freien Fettsäuren vor und nach der Inkubation unter Berücksichtigung der Leerwerte berechnet. Die Aktivitäten werden in Internationalen Einheiten (I. U. = μMol freigesetzte freie Fettsäuren/10^{10} Zellen/Minute) angegeben.

Histochemischer Esterasenachweis: Die luftgetrockneten Ausstriche werden 5 Minuten in Formoldampf fixiert, gespült und getrocknet. Die Präparate werden hierauf 60 Minuten bei Zimmertemperatur in der folgenden Lösung inkubiert: 80 mg Echtblausalz BB in 10 ml 0,1 m Phosphatpuffer (pH 6,8–7,0) gelöst und 0,4 ml Propylenglykol in 30 ml Puffer gelöst, dem tropfenweise 0,4 ml einer 1%igen Naphthol-AS-D-Acetat-Aceton-Lösung zugesetzt wird. Anschließend wird mit Leitungswasser gespült, 10 Minuten mit Mayers Hämalaun gefärbt, wieder gespült, gebläut und in Gelatinol eingebettet.

Tabelle I

Patient	Diagnose	Tributyrin	Tricaproin	Tricaprylin	Naphthol-AS-D-Acetat
H.	Osteomyelosklerose	3450	2820	2180	(+)
Ch.	Myeloische Reaktion	1690	1280	80	(+)
A.	Chronische Myelose	1310	830	155	(+)
B.	Chronische Myelose	3110	3520	–	+
N.	Chronische Myelose	6566	3180	443	+
Zo.	Akute Myelose	2580	1220	–	++
W.	Akute Myelose	1830	753		++
No.	Lymphadenose	1570	260	40	neg.
Z.	Lymphadenose	1490	466	–	neg.
H.	Lymphknotenzellen	1250	–		neg.
N.	Lymphosarkomzellen (Pleura)	6600	4300	1380	neg.
F.	Carcinomzellen (Pleuraerguß)	31500	–		+++
K.	Gaucherzellen	48700		–	+++
	Makrophagen (Maus)	14500			+++

Ergebnisse und Diskussion

In Tabelle I sind die gemessenen Enzymaktivitäten in verschiedenen weißen Blutzellen sowie Exsudatzellen und Gaucher-Zellen aus der Milz bei Verwendung von Tributyrin, Tricaproin

und Tricaprylin als Substrat angegeben. Alle untersuchten Zellen zeigten eine deutliche Esteraseaktivität. Die höchste Spaltungsgeschwindigkeit wiesen Gaucher Zellen aus der Milz, Zellen eines Uteruskarzinoms aus einem Pleuraexsudat und Mäusemakrophagen auf.

Die bisherigen Untersuchungen reichen nicht aus, um eventuelle quantitative Unterschiede zwischen normalen Granulozyten und Lymphozyten bzw. zwischen leukämischen Zellen nachzuweisen. Bei Verwendung von Triglyceriden längerkettiger Fettsäuren (Tricaproin und Tricaprylin) als Substrat zeigte sich allgemein eine Abnahme der Spaltungsgeschwindigkeit mit Zunahme der Kettenlängen der Fettsäuren.

Parallel zu den biochemischen Untersuchungen wurde der zytochemische Esterasenachweis an den gleichen Zellen unter Verwendung von Naphthol AS-D-Acetat als Substrat durchgeführt. Die Methode gestattet höchstens eine semiquantitative Beurteilung der Esteraseaktivität. Bei Vergleich der Ergebnisse fällt auf, daß sich in den Lymphozyten, im Gegensatz zu zytochemischen Methoden, biochemisch eine sichere, wenn auch nicht hochgradige Esteraseaktivität nachweisen läßt. Ansonsten ergibt sich eine recht gute Übereinstimmung zwischen Intensität der Esterasefärbung an fixierten Zellen im Ausstrich und der biochemisch gemessenen Esteraseaktivität von intakten funktionstüchtigen Zellen gegenüber Tributyrin.

In orientierenden Untersuchungen haben wir die Tributyrinaseaktivität von osmotisch zytolisierten oder mechanisch zerstörten Zellen untersucht. Es ergab sich in allen Fällen im Vergleich zu intakten Zellen ein Aktivitätsabfall von 10–50 %. Untersuchungen über die Aktivität einzelner Zellfraktionen sind im Gange.

Wie aus Tabelle II hervorgeht, wird Kokosfett sowohl von Granulozyten als auch von Lymphozyten hydrolytisch gespalten.

Tabelle II

Patient	Diagnose	Lipaseaktivität
Ch.	Myeloische Reaktion	160
A.	Chronische Myelose	92
N	Chronische Myelose	57
W	Akute Leukose	109
N.	Lymphadenose	55
H.	Lymphadenose	48
Makrophagen (Meerschweinchen)		910
		1040

Nach Inkubation dieser Zellen mit Glukose ohne Fettzusatz ist mit dieser Methode keine Zunahme der freien Fettsäuren festzustellen. Auf Grund der vorliegenden Untersuchungen ist demnach der sichere Nachweis geliefert, daß die weißen Blutkörperchen eine echte Lipaseaktivität besitzen. Dieser Befund erscheint uns von wesentlicher Bedeutung, da in letzter Zeit die Aufmerksamkeit in zunehmendem Maße auf die Rolle der Leukozyten im Fettstoffwechsel gelenkt wurde. So konnte JOCHIMS (8) die Fettaufnahme von Leukozyten nach Mahlzeiten zytochemisch wahrscheinlich machen, und IZAK UND DE VRIES (7) wiesen neben phasenoptischen Beobachtungen über die Fettaufnahme durch Leukozyten in biochemischen Untersuchungen nach, daß der Gehalt an gebundenen sowie insbesondere an freien Fettsäuren nach fettreichen Mahlzeiten in den Leukozyten stark zunimmt. Die Fähigkeit der Leukozyten zur Fettsäuresynthese aus Acetat 1 C^{14} ist erwiesen (3). Weiterhin könnten Lipasen beim Abbau von phagozytiertem Material eine wesentliche Rolle spielen (7).

Auch in bezug auf die Lipaseaktivität ergab sich bisher kein signifikanter Unterschied zwischen Granulozyten und Lymphozyten. Hingegen weisen Makrophagen eine höhere Aktivität auf. Da diesem Befund eine wesentliche funktionelle Bedeutung zukommt, wird er in einer folgenden Arbeit, nebst biochemischer Charakterisierung der Fermentaktivität, *ausführlich besprochen.*

Zusammenfassung

Die Spaltung von Triglyceriden mit kurzkettigen Fettsäuren (Tributyrin, Tricapron und Tricaprylin) sowie von Kokosfett durch weiße Blutzellen, Exsudatzellen und Gaucherzellen wurde untersucht. Parallel dazu wurde zytochemisch der Esterasenachweis mit Naphthol-AS-D-Acetat als Substrat geführt. Im Gegensatz zu den zytochemischen Befunden weisen Lymphozyten eine deutliche Esteraseaktivität auf. Ansonsten findet sich eine gute Übereinstimmung zwischen biochemischen und zytochemischen Befunden. Mit Zunahme der Kettenlänge der Fettsäuren der verwendeten Substrate nimmt die Spaltungsgeschwindigkeit ab. In normalen und pathologischen Granulozyten und Lymphozyten wird unter Verwendung von Kokosfett als Substrat eine deutliche Lipaseaktivität nachgewiesen. Zwischen diesen beiden Zellarten lassen sich bisher keine quantitativen Unterschiede nachweisen. Makrophagen weisen eine bedeutend höhere Aktivität auf. Die physiologische Bedeutung dieser Befunde wird kurz diskutiert.

Summary

The authors studied the cleavage of triglycerides with short-chained fatty acids (tributyrine, tricaproine and tricaphylline) and of coconut oil by leukocytes, exudate cells and Gaucher's cells. Parallel cytochemical esterase determinations with Naphthol-AS-D acetate as substrate were performed. In contrast to the cytochemical findings,

lymphocytes show an evident esterase activity. Otherwise the agreement between the biochemical and cytochemical results is good. As the chain of the fatty acids in the substrate lengthens, the cleavage rate decreases. In normal and pathological granulocytes and lymphocytes, using coconut oil as substrate, marked lipase activity is found. Quantitative differences between these two cell forms have not been demonstrated so far. The physiological significance of these findings is discussed.

Résumé

Etude de la scission de triglycérides formés d'acides gras à courtes chaines (tributyrine, tricaproine et tricapryline) et de graisse de coco par les leucocytes, les cellules d'exsudat et les cellules de Gaucher. Détermination parallèle de l'estérase par méthode histochimique avec comme substrat l'acétate de AS-D-naphtol. Les lymphocytes montrent une nette activité estérasique qui n'est cependant pas confirmée par l'histochimie. A part cette exception, il existe une bonne corrélation entre les déterminations biochimiques et histochimiques. La vitesse de scission diminue avec la longueur des chaines des acides gras employés comme substrats. L'activité lipasique des granulocytes et des lymphocytes normaux et pathologiques est nette lors de l'emploi de la graisse de coco comme substrat. Aucune différence d'ordre quantitatif n'a pu être démontrée entre ces deux types cellulaires. Les cobbayes montrent une plus grande activité. La signification physiopathologique de ces particularités est discutée.

Literatur

1. Ammon, R.: In Ammon's und Dirscherl: Fermente, Hormone, Vitamine, Band I, pp. 99–191 (Georg Thieme Verlag, Stuttgart 1959).
2. Bergel, S.: Fettspaltendes Ferment in den Lymphozyten. Münch. med. Wschr. 56: 64–66 (1909).
3. Buchanan, A. A.: Lipid synthesis by human leukocytes in vitro. Biochem. J. 75: 315–320 (1960).
4. Dole, V. P. and Meinertz, H.: Microdetermination of long-chain fatty acids in plasma and tissues. J. biol. Chem. 235: 2595–2599 (1960).
5. Frei, J.; Borel, C.; Horvath, G.; Cullity, B. and Vannotti, A.: Enzymatic studies on the different types of normal and leukemic human white cells. Blood 18: 317–327 (1961).
6. Hardin, E. B.; Valentine, W. N.; Follette, J. H. and Lawrence, J. S.: Esterase and lipase activity of leukocytes and erythrocytes in health and disease. Amer. J. med. Sci. 229: 397–403 (1955).
7. Izak, G. and de Vries, A.: Studies on lipophagocytosis. I. Lipophagocytosis by human white blood cells in vitro and in vivo. J. Lab. clin. Med. 55: 564–583 (1960).
8. Jochims, J.: Leukozyten und Fetttransport. Beobachtungen an Kindern im Hunger und im Coma diabeticum. Klin. Wschr. 40: 520–527 (1962).
9. Löffler, H.: Cytochemischer Nachweis von unspezifischer Esterase in Ausstrichen. Beiträge zur Technik und Ergebnisse im Blutausstrich des Menschen. Klin. Wschr. 39: 1220–1227 (1961).
10. Neri, F.: Über die lipolytische Fähigkeit der weißen Blutkörperchen. Biochem. Z. 124: 156–164 (1921).
11. Sarda, L.; Marchis-Mouren, G. et Desnuelle, P.: Séparation et différenciation des activités estérasiques et lipasiques du pancréas. In Desnuelle: The Enzymes of Lipid Metabolism, pp. 20–21 (Pergamon Press, Oxford/London/New York/Paris 1961).

Adresse der Autoren: Dr. H. Braunsteiner, Dr. F. Dienstl, Dr. S. Sailer und Dr. F. Sandhofer, II. Med. Univ.-Klinik, Garnisongasse 13, Wien IX (Österreich).

Acta haemat. 30: 340–343 (1963)

Institute of Clinical Biochemistry and Pediatric Department, University Hospital, Oslo

Studies of Patients with Familial Vitamin B_{12} Malabsorption

The Lack of Effect of Normal Intestinal Juice on the Absorption of Vitamin B_{12}

By PÅL BJÖRNSTAD AND OLGA IMERSLUND

Recently we have described 10 cases of a familial and apparently congenital malabsorption of vitamin B_{12} (4 5) in which none of hitherto known causes of vitamin B_{12} malabsorption could be demonstrated. It was evident that the defect in these patients was located at the level of the small intestine, and was due to an interference with the transport of vitamin B_{12} across the intestinal wall. In the previous papers we have suggested that congenital lack of a specific intestinal factor might be responsible for the malabsorption of vitamin B_{12}.

COOPER AND CASTLE (2) have demonstrated a factor present in extracts of intestinal wall from rats which in vitro splits the binding of vitamin B_{12} to intrinsic factor HERBERT (3) by the same technique has demonstrated a similar factor in extracts from human intestinal wall.

The purpose of the present work has been to test the effect of normal intestinal juice on the absorption of vitamin B_{12} in some of our previously described patients in an attempt to evaluate if a factor such as that described by COOPER AND CASTLE is present in intestinal juice, and furthermore to investigate if the juice may cause increased vitamin B_{12} in our patients.

While this work was in progress, COLLE et al. (1) have described a case which both clinically and with regard to pathogenesis seems to belong to the same category as our patients. They demonstrated increased absorption of radioactive vitamin B_{12}, when the test dose was given together with normal intestinal juice.

Material

The material consists of 6 patients, 3 males and 3 females, aged 7–19 years. Receiving monthly injections of 50–100 µg. vitamin B_{12} they were in good condition and in haematological remission. Their complete case histories and the laboratory findings are described in the earlier papers (4, 5). In the present paper they have the same designation as before with roman numbers. The patients are Nos. II, VI, VII, VIII, IX and X. Patients Nos. VII and VIII are siblings, the others are from different families and not related to each other.

Methods

The absorption of radioactive vitamin B_{12} has been measured by the method of SCHILLING (7) as described in our previous communication (5).

Intestinal juice was collected from patients with surgical fistulas (jejunostomy or ileostomy because of colectomy). The juice was diluted with half the volume of 0.9% solution of NaCl and filtered. It was stored deep frozen at −20 °C until just before use.

In one experiment (patient No. IX) we also tested extract from the small intestine of hog: The mucosa layer of the small intestine was homogenized in a Waring blendor with about 300 ml of 0.9% solution of NaCl and filtered.

As the intestinal factor possibly lacking in our patients most likely is an enzyme, it was anticipated that the protein might be destroyed by the pepsin containing gastric juice. To avoid this possibility the juice was administered through a long Ryle tube. The position of the tube was controlled by fluoroscopy or X-ray. In 5 of the 7 experiments the tip of the tube was placed at the duodenojejunal flexure or below, and in the 2 others in the stomach. The administration of the juice was started 30 minutes after the testdose of radiolabelled vitamin B_{12} had been given, 60–70 ml were administered slowly in the course of 30 minutes.

Table I

Results of the SCHILLING Test.

Effect of normal intestinal juice and hog intestinal extract.

Pat. No.	Age, years	Position of tip of the tube	SCHILLING test (% excretion of ^{60}Co B_{12} in 24 hours urine) without juice	Administration of juice from	SCHILLING test (% excretion of ^{60}Co B_{12} in 24 hours urine) after juice
II	7	Duodenojejunal flexure	1.3	Ileostomy	0.3
VI	14	Duodenojejunal flexure	0.2	Ileostomy	0.3
VII	19	25 cm below duodenojejunal flexure	0.1	Jejunostomy	0.4
VIII	14	25 cm below duodenojejunal flexure	0.2	Ileostomy	0.2
IX	12	Stomach	1.4	Hog intestinal extract	0.5
IX	12	Stomach	1.4	Ileostomy	0.5
X	11	Duodenojejunal flexure	0	Ileostomy	0.2

Results and Discussion

As shown in table I we were not able to demonstrate significant increase in the urinary excretion of ^{58}Co-labelled vitamin B_{12}, when the testdose was given either with normal intestinal juice or extract of small intestine from hog. Thus, there was no evidence that normal intestinal juice or hog intestinal extract increased the *absorption of vitamin B_{12} in our patients. However* in spite of the negative results it might well be that our patients are lacking a factor such as that described by COOPER AND CASTLE (2) in extract from intestinal wall. It is reasonable to assume that the factor is located intracellularly and not secreted in the intestine.

The patient reported by COLLE et al. (1) was a 26 months old female. They also demonstrated the defect to be placed at the level of the small intestine. When the SCHILLING test was performed in the standard manner or together with intrinsic factor they found an urinary excretion of less than 1% except on one occasion, when 8.3% was excreted in 48 hours. Performing the test with both intrinsic factor and intestinal juice, they found an excretion of 4.3%, 11.7% and 0.2% on three different occasions. To evaluate the effect of normal intestinal juice on the absorption of vitamin B_{12} out of these experiments seems rather difficult, but it is evident that the juice was effective at least on one occasion. The fact, that the juice was ineffective in our experiments, may be due to a different biochemical defect in our patients.

In our opinion the complete investigation of vitamin B_{12} malabsorption of obscure etiology should include experiments with *normal intestinal juice in an attempt to demonstrate* if different pathogenic groups exist.

Addendum. After the completion of this paper MOLLIN et al. (6) have reported 2 cases of acquired vitamin B_{12} malabsorption in which none of the hitherto known causes of vitamin B_{12} malabsorption could be demonstrated. The patients were 62 years old woman and 67 years old man. They demonstrated significant increase in the absorption of vitamin B_{12}, when the testdose was given together with succus entericus. These findings support the view that different pathogenetic groups must exist.

Summary

In 6 cases of familial the administration of normal intestinal juice or hog intestinal extract vitamin B_{12} malabsorption had no effect on the absorption of vitamin B_{12}, measured by the method of SCHILLING.

Résumé

Chez 6 patients souffrant d un trouble de la résorption intestinale de la vitamine B_{12}, l'administration de suc intestinal normal ou d'extrait d'intestin de porc influence pas cette résorption, appréciée par le test de SCHILLING.

Zusammenfassung

Bei 6 Patienten mit einer familiären Störung der Vitamin B_{12}-Resorption vermochte die Zufuhr von normalem Darmsaft oder Extrakt aus Schweinedarm die Resorption von Vitamin B_{12}, gemessen mit dem SCHILLING Test, nicht zu beeinflussen.

References

1. COLLE, E., GREENBERG, L. AND KRIVIT W. Studies of patient with selective deficiency in absorption of vitamin B_{12}. Blood *18*: 48 (1961).
2. COOPER, B. A. AND CASTLE, W. B. Sequential mechanisms in the enhanced absorption of vitamin B_{12} by intrinsic factor in the rat. J. clin. Invest. *39*. 199 (1960)
3. HERBERT V. Mechanism of absorption of vitamin B_{12}. Fed. Proc. *19*: 884 (1960)
4. IMERSLUND, O. Idiopathic chronic megaloblastic anemia in children. Acta paediat., Suppl. *II* (1960).
5. IMERSLUND, O. AND BJØRNSTAD, P. Familial vitamin B_{12} malabsorption. Acta haemat. *30* 1 (1963).
6. MOLLIN E. R.; MANGUM, J. F.; COHEN, J. L. AND PORTER, W. R. Selective malabsorption of vitamin B_{12}. Amer. J. med. Sci. *245*. 699 (1963)
7. SCHILLING, R. F. Intrinsic factor studies. II. The effect of gastric juice on the urinary excretion of radioactivity after the oral administration of radioactive vitamin B_{12}. J. Lab. clin. Med. *42*: 860 (1953)

Authors' address: Dr. P. Bjørnstad and Olga Imerslund, Rikshospitalet, Oslo (Norway).

Acta haemat. 30, 344–351 (1963)

From the Institute of Clinical Medicine, University of Freiburg i. Br.
(Director: Prof. L. Heilmeyer)
Department of Immunopathology (Prof. H. Schubothe)

The Antigenic Components of Elinin

I. Isolation and Serological Identification

By Nicoletta Vulpis

The antigenic composition of red blood cell S protein has been the subject of repeated investigations. The results of these works have been reported in several communications (5, 6, 7). It is certain that S protein is not the only protein in the red cell wall; another water soluble fraction, denoted as *elinin*, was also derived from red cell membranes by Moskowitz et al. (3). Elinin differs from S protein in having less N and more P. It is a lipo-protein fraction, which is defined essentially by a method of preparation rather than by a set of exact physical and chemical properties. However, the fact that this material does possess certain characteristic properties has led Moskowitz to give it a specific name.

Immunological methods are proving increasingly useful for exploring the homogeneity or heterogeneity of proteins. In general these methods are more sensitive than physical methods for detecting heterogeneity. This report describes the application of immunochemical methods to the characterization of the elinin and its differentiation from S protein.

Methods

Preparation of elinin. The method for recovering elinin (EL) used in the present study is based on the technique outlined by Moskowitz et al. (3). An air-driven ultracentrifuge manufactured by Phywe in Göttingen was used. The water solution of EL was obtained from ORh positive red cells of normal donors; it was prepared as 500 mg./100 ml. solution and used as the source of antigens for the preparation of the immunization [illegible] and immunochemical methods.

Production of rabbit antisera. The antigen was mixed with an equal volume of Freund's Adjuvant (complete)* until a homogeneous suspension was obtained. One ml. of the elinin adjuvant emulsion was injected intramuscularly into each thigh of tw

---

This work was carried out while the author was a NATO grantee in Germany.

* Commercial product purchased from Difco, Detroit.

rabbits one each week for 6 weeks (0.5 mg. protein/dose). Rabbits were bled periodically from the marginal ear vein and the sera collected for precipitin testing. When sufficiently high titers were obtained, the rabbits were bled from the heart by cardiac puncture and the serum were recovered and either frozen at −70 C or preserved 4 C in the presence of 0.01% thimerosal. The antisera were classified as anti$_1$ and anti$_2$-EL.

The rabbit anti-human serum was obtained from Behringwerke AG, Marburg Lahn.

The antiserum against human stromata was prepared was described in the previous paper (5).

Determination of antiserum potency. Qualitative evaluation of the strength of the antisera was made with tw different micro-ring precipitin tests, the antigen dilution method and the more classical serum dilution titer (2). The antigen was the same as employed to induce antibody formation in the rabbit.

I the antigen dilution method the antigen was serially diluted from 0.5 solution and used against undiluted antisera. Micro-tubes made from glass tubing, internal diameter 3 mm were used. Equal volumes (0.025 ml) of antigen and antisera ere employed and the precipitation reaction developed at room temperature (25 C. Simultaneously the rabbit normal serum (before inoculation) was run as control. The formation of precipitin bands was checked t ½, 1 2, 4 and 24 hours. The end point was considered to be barely detectable ring at the smallest amount of antigen (about 1 µg) to produce clearly visible ring in the previously described test.

Absorption technic: Antisera were absorbed with normal human serum, crystalline human haemoglobin or elinin. This was carried out by mixing the serum with an excess of absorbent antigen. The antiserum-antigen mixture was left at room temperature for 30 minutes. The resulting precipitate was removed by centrifugation and the clear supernatant was left in the refrigerator at 4 C for 4 days to permit complete neutralization of the corresponding antibodies. The absorbed antiserum was tested against the absorbent antigen by the precipitin test. The absorption was considered complete if no reaction took place.

Precipitin studies. The quantitative precipitin determinations were conducted by the method of HEIDELBERGER AND KENDALL (1) as described by KABAT AND MAYER (2). The analyses were carried out by adding 0.25 ml of undiluted absorbed antiserum to each of series of tubes containing increasing amounts of EL. The contents were immediately mixed and stored 1 h at 37 C and then 2 days at 4 C. The precipitates were centrifuged at 2 C and washed twice with cold saline. Nitrogen contents of the precipitates ere determined by the micro-Kjeldahl method. The results are recorded on the basis of total N precipitated.

Ouchterlony analyses: The double diffusion-in-gel method of OUCHTERLONY was used in the micromodification of WADSWORTH (8).

Immunoelectrophoretic analyses: The microtechnique described by SCHEIDEGGER (4) was used. A standard microscope slide (16 28 mm) was covered with 2 ml. warm buffered agar solution. 2% Difco Bacto-Agar purified as described in an earlier communication (6) was made up in barbital-hydrochloric acid buffer at pH 8.6. The immunodiffusion electrophoresis equipment was manufactured by Egaton Aktiengesellschaft, Bern, Switzerland. Electrophoresis was carried on with the aforementioned buffer at pH 8.6 for 30 minutes using 40 volts (accross the slides) and 40 mA. The agar coated slides were washed with 0.9% saline and remained in distilled water bath overnight. The dried slides were then stained with amidoschwarz 10 B for 15–20 minutes.

Results

Quantitative precipitin tests Table I and fig 1 show the data obtained when increasing amounts of antigen were added to a

series of tubes containing a constant volume of antiserum. Determinations were usually carried out in duplicate and the figures in the table represent averages. Portions of each supernatant were tested by addition of antigen and antiserum to determine whether excess of antibody or antigen was present The first and second column in the table show the amount nitrogen added and the total (antigen + antibody) nitrogen precipitated (fig 1 curves 1 and 2)

Table I

Addition of increasing amounts of elmin to homologous rabbit antisera.

Elmin N added mg	Total N pptd. mg	Tests on supernatants For antibody	For antigen
	serum anti₁-EL		
0.002	0.002	++++	±
0.006	0.012	++++	±
0.012	0.020	+++	+
0.022	0.022	+++	+
0.060	0.036	+	+
0.120	0.043	±	+
0.150	0.042	—	++
0.200	0.030	—	+++
	serum anti₂-EL		
0.002	0.004	++++	±
0.006	0.022	+++	±
0.012	0.032	+++	+
0.022	0.052	++	++
0.060	0.090	+	+++
0.120	0.109	+	+++
0.150	0.135	+	+++
0.200	0.120	+	++++

It can be seen that with increasing additions of EL, the total nitrogen precipitated rose to a maximum of 0 043 mg when 0 120 mg of EL nitrogen was added to 0.25 ml of anti$_1$ EL serum, as compared to 0 135 mg obtained with the anti$_2$ EL serum. The absence of an equivalence zone, observed in the tests on supernatants from the precipitates, showed that most likely EL was serologically heterogenous.

Precipitin tests in agar In double diffusion in-gel analyses EL formed one precipitate with the immunserum anti$_1$ EL and two parallel precipitates with anti$_2$ EL serum (fig 2) One of these gave a reaction of identity with the precipitate obtained with anti$_1$ EL, the other one gave on the contrary a reaction of interference The same figure shows that both normal rabbit serum and rabbit anti human serum did not form precipitin lines with EL. However

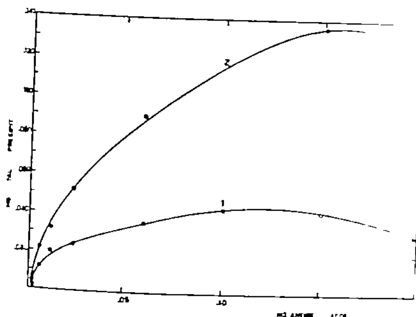


Fig. 1 Precipitin curves for EL and its homologous antibody. Curve 1 refers anti$_1$ EL immune serum, curve 2 to anti$_2$-EL immune serum.



Fig. 2. Micro-Ouchterlony agar-diffusion system. 10 µg EL in central well. 1) anti$_1$-EL immune serum; 2) anti$_2$-EL immune serum 3) normal rabbit serum 4) rabbit anti-human serum. Wells 1–4 contain 22 µl of serum. Diffusion allowed to proceed for 18–24 hours at room temperature.

when the serum anti$_2$ EL was compared with EL as well as with human normal serum (HNS) and human normal haemoglobin (HNHb) the same two parallel precipitates were obtained with HNS presumably due to the small amount of HNS in the EL preparations (fig. 3a). After absorption with HNS this band disappeared. Human haemoglobin did not show any precipitin reaction (fig 3b). Absorption studies using EL completely neutralized the activity of the serum.

of antigen. The shape of the EL precipitin band suggested several components in EL. Comparative immunoelectrophoretic studies showed that anti, EL serum did not give any precipitate with HNHB whereas gave a precipitin band with HNS in the gamma-globulin zone (fig 5c) The absorbed immunserum was then unable to form a precipitate.

Discussion

Elinin was an effective antigen in eliciting antibody formation in rabbits. Specific antisera and precipitin reactions in agar were used to assess its degree of purification These studies showed that El contains at least two distinct antigenic fractions which are present in quantities sufficient to stimulate antibody formation in rabbits immunized with the aid of Freund s adjuvant.

The serologic heterogeneity of EL is also confirmed by the results of the immunoelectrophoretic analysis. In fact, the long arc of only moderate curvature suggests that it was formed by an antigen which electrophoretically is heterogeneous and consists of a population of molecules with smoothly graduated differences in electrophoretic mobility Thereafter the EL solution contained serologically detectable impurities derived from the ghosts from which it was prepared. Traces of serum were showed and the antibodies to these associated proteins were absorbed from the immunserum by human serum. It would seem very likely that very small amounts of haemoglobin were present in the FL preparations, even though they were not detected.

The data in this report show also that EL is distinct from the other recognized protein of red cell membrane that is S protein. Comparative studies of these two proteins showed that using a rabbit anti-stromata serum EL gave a reaction of interference with the precipitates formed by S protein.

Acknowledgements. The author wishes to express her sincere appreciation for the hospitality of Prof. L. Heilmeyer and Prof. H. Schubothe, whose constant advice and encouragement ha e been great stimulation to this work. The author is also grateful to Prof. H. Schubothe for his help in solving the problems of instrumentation and to Dr T Luthardt of the Department of Pediatrics for the use of the ultracentrifuge Many thanks are due t Miss Schmidt for her excellent technical assistance in operating the ultracentrifuge

Summary

Immunochemical methods were used in order to investiga the antigenic structure of elinin obtained from human normal red cell membranes. By means of rabbit sera raised against elinin two major immunologically identifiable fractions ere found. In

comparative immunoelectrophoretic studies single long arc in the alpha₂-zone was obtained. Elinin was distinguished immunologically from the other previously recognized protein of red cell ghosts, S protein.

Résumé

L'auteur étudie avec une méthode immunochimique la structure antigénique de l'élinine des membranes érythrocytaires humaines normales. A l'aide de sera anti-élinine de lapins on retrouve deux fractions antigéniques. L'immunoélectrophorèse comparative révèle une ligne de précipitation courbe et étirée dans la zone des alpha₂. L'élinine se distingue immunologiquement de la protéine S décelée auparavant dans le stroma érythrocytaire.

Zusammenfassung

Die Antigenstruktur von Elinin aus Membranen normaler menschlicher Erythrozyten wurde immunochemisch untersucht. Mit Hilfe von Seren gegen Elinin immunisierter Kaninchen wurden zwei immunologisch nachweisbare Fraktionen gefunden. Die vergleichende Immunelektrophorese ergab eine langgestreckte Bogenlinie in der α_2-Zone. Elinin unterscheidet sich immunologisch von dem früher nachgewiesenen S-Protein des Erythrocytenstromas.

References

1. Heidelberger, M. and Kendall, F. E. Precipitin reaction between type III pneumococcus polysaccharide and homologous antibody theory of reaction mechanism. J. exp. Med. 61: 563–591 (1935). – Quantitative theory of precipitin reaction study of azoprotein-antibody system. J. exp. Med. 62: 467–483 (1935). – Quantitative theory of precipitin reaction reaction between crystalline egg albumin and its homologous antibody J. exp. Med. 62: 697–720 (1935).
2. Kabat, E. A. and Mayer, M. M. Experimental Immunochemistry 2nd ed. (C C Thomas, Springfield, Ill. 1961).
3. Moskowitz, M. Dandliker, W. B.; Calvin, M. and Evans, R. S. Studies on the antigens of human red cells. I. The separation from human erythrocytes of water soluble fraction containing the Rh, A and B fractions. J Immunol. 65: 383–392 (1950).
4. Scheidegger, J. J. Une micro-méthode de l'immuno-électrophorèse. Int. Arch. Allergy 7: 103–110 (1955).
5. Vulpis, N. Studies on the antigens of human red cell ghosts. Differences in the S proteins of the adult and the new-born. Acta haemat. 30: 220–233 (1963).
6. Vulpis, N. Studies on the antigens of human red cell ghosts. Differences in antigenic specificity of human normal adult, hereditary spherocytosis and auto-immune haemolytic anaemia S protein. Acta haemat. 30: 280–287 (1963).
7. Vulpis, N. et Vulpis, G. Etudes sur la constitution antigénique du stroma normal par des méthodes, de précipitation spécifique en milieu gélifié et d'immuno-électrophorèse. Constitution antigénique de la protéine S. Nouv. Rev. franç. Hémat. 2: 535–550 (1962).
8. Wadsworth, C. A microplate technique employing gel chamber compared with other micro- and macroplate techniques for immune-diffusion. Int. Arch. Allergy 21: 131–137 (1962).

Author's address: Dr. Nicoletta Vulpis, Via de Rossi 22, Bari 2/8 (Italy).

International Review of Tropical Medicine. Edited by *David Richard Lincicome.* Vol. 2, 1963. Academic Press Inc., New York, N. Y. 425 p. Price: $ 16.00.

Most of the articles in this volume concern tropical medicine, but the following are of interest for haematologists: *Abnormal Hemoglobins and Thalassemia in Asia* are discussed by F. Vella (Singapore); abnormal variants of hemoglobin A and F are common in Asia. In the western parts S is most frequent, in the northern parts of Asia E is the most common. – R. S. Bray (Harbel, Liberia) contributes an article on the "Exoerythrocytic Phase of Malaria Parasites" which is now considered by malaria experts of great importance for the occurrence of relapses. – H. D. Barnes (Johannesburg, South Africa) writes briefly on Porphyria. – Charmingly written is a chapter by Sir Philip Manson-Bahr entitled "*The Story of Malaria: The Drama and the Actors*". It brings many personal and other not generally known details, short sketches of the classical researchers of Malaria who were the protagonists and main discoverers. Their "foibles and quibbles and the rivalries" are here amply documented.

G. Rosenow, New York

Index rerum ad Vol. 26–30

Bearbeitet von G. Bodmer, Basel

(B) = Buchbesprechungen – Livres nouveaux – Book reviews

Index autorum ad Vol. 26–30

(B) = Buchbesprechungen – Book reviews – Livres nouveaux